UNIVERSITY OF ALBERTA

REDUCING METHANE EMISSIONS FROM RUMINANT ANIMALS

BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **REDUCING METHANE EMISSIONS FROM RUMINANT ANIMALS** submitted by **YAN DONG** in partial fulfilment of the requirements of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL NUTRITION.**

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ABSTRACT

In vitro and in vivo experiments were conducted to determine the effectiveness of lipids, exogenous fibrolytic enzymes, and the specific anti-methanogenic agent α-bromoethanesulfonic acid (BES) in reducing methane emissions from ruminant animals and their effect on dietary digestion. A rumen simulation technique system equipped with eight fermenters was used for in vitro investigations. Six ruminally and duodenally fistulated ewes were used for in vivo investigations.

Coconut, canola and cod liver oils (10% of diet, w/w) reduced ($P < 0.05$) methane emissions by 59, 26 and 29%, respectively, with a hay diet in in vitro studies. Comparative values with a concentrate diet were 85, 32 and 49% ($P < 0.05$). Coconut oil depressed ($P < 0.05$) dry matter (DM) digestion of the hay diet by 31%, but it had no influence on DM digestibility of the concentrate diet. Neither canola nor cod liver oil had any detrimental effect on the digestion of diets. Exogenous fibrolytic enzymes (4% of diet, wt/wt) increased ($P < 0.01$) methane emissions by 43% and organic matter (OM) digestibility by 9%. α-bromoethanesulfonic acid (25 μM) depressed ($P < 0.01$) methane emissions by up to 52% without a detrimental effect on digestion.

In the study with sheep, exogenous fibrolytic enzymes (1.5% of diet, vol/wt) or BES (200 μM) did not affect digestion, ruminal digestive kinetics, or methane emissions. Methane emissions (g/kg DMI) varied with intake of individual ewes, being about 20% lower ($P < 0.05$) at DM intake of 1.5 vs 0.6 kg/d. Similarly, methane emission (g/kg DMI) was decreased ($P < 0.01$) by 26% when apparent DM digestibility of the grass hay in the total tract increased from 36 to 57%. Diaminopimelic acid outflow to the duodenum (g/kg

ruminally digested OM) and methane emissions (g/kg ruminally digested OM) decreased ($P < 0.0001$) by 67 and 64%, respectively, when apparent OM digestibility of grass hay in the rumen of ewes increased from 16 to 40%.

It was concluded that supplementation of lipids and BES effectively depressed methane emissions in vitro, but BES had no influence on methane emissions in sheep. Exogenous fibrolytic enzymes effectively increased forage digestion and methane emissions in vitro, but it had no effect on either forage digestion or methane emissions in sheep. Further studies are required with a more potent anti-methanogenic agent(s) to examine the relationship between methanogensis and microbial growth and dietary fermentation in the rumen

TO MY PARENTS AND MY WIFE FOR THEIR LOVE AND ENCOURAGEMENT

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LIST OF ABBREVIATIONS

ADF	Acid detergent fibre
ATP	Adenosine 5'-triphosphate
BES	α-Bromoethanesulfonic acid
CoM	Coenzyme M
CP	Crude protein
DAPA	Diaminopimelic acid
DE	Digestible energy
DM	Dry matter
DMI	Dry matter intake
FBL	Feed bag liquid
GE	Gross energy
ISDMD	In situ dry matter disappearance
*k*p(L)	Constant of rumen liquid dilution rate
*k*p(P)	Constant of rumen particulate matter passage rate
ME	Metabolizable energy
NADH	Nicotinamide adenine dinucleotide
NDF	Neutral detergent fiber
OM	Organic matter
OBR-HEC	Ostrazin brilliant red-hydroxymethyl cellulose

RBB-xylan	Remazol brilliant blue-xylan
RDMD	Ruminal DM digestibility
RDNDF	Ruminal digested neutral detergent fiber
RDOM	Ruminal digested OM
RTDOM	Ruminal truly digested OM
RUSITEC	Rumen simulation technique
SEM	Scanning electron microscopy
TDMD	True DM digestibility
VFA	Volatile fatty acid

LIST OF UNITS

atm	Atmosphere
°C	Degree Celsius
d	Day
h	Hour
kg	Kilogram
mg	Milligram
µg	Microgram
L	Liter
mL	Milliliter
µL	Microliter
IU	International unit
Kcal	Kilocalorie
M	Molar
mM	Millimolar
µM	Micromolar
mV	Millivolt

1. INTRODUCTION

1.1. Background

The digestive system of ruminant allows the animal to live on fibrous biomass such as roughages, cereal straws and other waste products, which are converted to high quality protein for human consumption. Ruminant animals, in contrast with monogastric animals, do not need to compete with humans for grain in their diets. This characteristic gives ruminant animals a unique position in the world economy. In recent years, however, ruminant production has caused concern with respect to their contribution to environmental problems, not only in terms of solid and liquid animal waste products, but also because of excretion of considerable amounts of carbon dioxide (CO_2) and methane (CH_4). It has been well documented that increased concentrations of CO_2 and CH_4 gases in the atmosphere are closely associated with global warming. This phenomenon is called as the greenhouse effect. It is estimated that the global temperature will continuously increase by 1.5 to 4.5°C over the next 50 years if emissions of greenhouse gases are not controlled (Environment Canada 1988). Global warming is predicted to cause erratic weather patterns, melting glaciers, flood, and drought.

Carbon dioxide is the single greatest contributor to the greenhouse effect. The concentration of CO_2 is increasing at an annual rate of 5% and has increased by 25% over the past 200 years (Environment Canada 1991).

Methane is deemed to be the second most important greenhouse gas after CO_2,

contributing approximately 25% as much to global warming as CO_2 (Tyler 1991). The concentration of CH_4 in the atmosphere has increased in recent history with the higher levels corresponding to periods of higher temperature and deglaciation. The atmospheric CH_4 concentration was approximately 0.7 ppm (by volume) 10,000 years ago until about 300 years ago (Tyler 1991). Since that time, CH_4 in the atmosphere has increased steadily at an annual rate of 1% until it has reached a value of 1.7 ppm in the early 1990's (Thompson et al. 1992; Moss 1993). Although the atmospheric concentration of CH_4 is considerable lower than that of CO_2 (~ 345 ppm), the increase in atmospheric CH_4 causes great concern because it has a higher infrared absorbance, thus per molecule it is considered to be 30 times more effective a greenhouse gas than CO_2 (Tyler 1991). In addition, CH_4 in the stratosphere reacts with free radicals, mainly hydroxyl radical, forming $CH_3\cdot$ which can then participate in a number of complex reactions leading to ozone destruction (Tyler 1991).

It is estimated that ruminants in the world are responsible for approximately 77×10^6 metric tonnes of CH_4 production annually, which constitutes approximately 15% of the total atmospheric CH_4 emissions (Crutzen et al. 1986; Lashof and Tirpak 1990; Moss 1993). Others have speculated that domestic animals are responsible for 12-30% of the total CH_4 production (Anonymous 1990; Crutzen 1995). Ruminants produce approximately 97% of CH_4 generated by domestic animals (McAllister et al. 1996). Given a world population of approximately 1.3 billion cattle and calves, each animal producing about 44 kg of CH_4 annually, cattle account for approximately 75% of total CH_4 emissions from domestic animals (Crutzen et al. 1986). The global production of CH_4 from cattle and sheep has been increasing by approximately 1% annually (Crutzen et al. 1986).

In addition to a significant contribution to global warming, CH_4 produced by ruminants represents a serious diversion of dietary energy away from animal growth and production. It is estimated that 2-15% of gross energy in the feed can be lost through CH_4 formation in the rumen (Van Nevel and Demeyer 1996). Thus, reducing methane production in ruminants would be beneficial to both ruminant livestock industry and the atmosphere. However, methanogenesis plays a very important role in anaerobic fermentation. Methanogens interact mutualitically with fermentative microorganisms in the rumen and other anaerobic habitats allowing disposal of electrons, thereby promoting growth of fermentative microorganisms and degradation of organic matter. Previous studies have provided evidence that drastic inhibition of methanogenesis can cause a depression in anaerobic fermentation of organic matter (Johnson and Johnson 1995; Mathison 1997). However, alternative hydrogen sinks are present in the rumen (Mathison et al. 1998). Some ruminal microbes can alternatively dispose of electrons through pathways of ethanol, lactate and propionate synthesis (Wolin and Miller 1988). In addition, metabolic hydrogen can be utilized in the reduction of inorganic substrates such as nitrate and sulfate (Czerkawski 1986). These would suggest that ruminal methanogenesis may be able to be controlled without a detrimental effect on microbial growth and organic matter fermentation by proper manipulation of ruminal fermentation.

Inhibition of methane emissions in the rumen by long-chain unsaturated fatty acids has been recognized for many years, but previous reports about the effects of long-chain fatty acids on dietary digestibility are not consistent. Canola oil is sometimes used as a high-energy supplement to finishing diets of feedlot cattle in western Canada. The first in vitro

experiment of this thesis research was conducted to compare canola oil with cod liver oil and coconut oil, which are different in fatty acid profiles, on microbial populations, methane emissions and feed digestibility. A hay diet and a wheat-based diet were used to determine the effect of these oils on both forage and concentrate diets.

The second in vitro experiment was undertaken to examine the effect of exogenous fibrolytic enzymes and α-bromoethanesulfonic acid (BES) on microbial populations, methane emissions and diet digestibility. Pretreatment of forages with exogenous fibrolytic enzymes has been shown to improve fibre digestion in vivo and in vitro, but studies concerning their effect on methane emissions is limited. α-Bromoethanesulfonic acid, a specific methanogenic inhibitor, has been shown the potential to inhibit methanogenesis in pure cultures and mixed rumen microbial cultures as well as in sheep, but its effect on diet digestibility has not been examined.

Finally, an in vivo study with sheep was conducted to confirm the results obtained from the second in vitro experiment and to study the relationships between methane emission, bacterial growth, and the digestion of a diet consisting of fescue hay.

1.2. Hypotheses

It was hypothesized that:

1. Depression in ruminal methanogenesis would not have a detrimental effect on ruminal digestion of organic matter because many rumen microbes have an alternative mechanism(s) to regenerate NAD^+;

2. Canola oil would reduce ruminal methanogensis without a detrimental effect on ruminal fermentation of organic matter because the double bonds of long-chain unsaturated fatty acids of canola oil can function as an alternative means of disposing metabolic hydrogen or long-chain fatty acids can be toxic to methanogens;

3. Exogenous fibrolytic enzymes would reduce ruminal methanogensis because they can increase the competitiveness of ruminal lactate- and propionate-synthesizing bacteria with acetate-producers by accelerating release of simple sugars from fibrous components of forages; and

4. α-Bromoethanesulfonic acid would inhibit ruminal methanogenesis because it is a competitive inhibitor of coenzyme M (CoM) which is a prerequisite enzyme for methane synthesis.

1.3. Objectives

The major objective of this research was to develop strategies to reduce methane production in the rumen. To accomplish this objective, several projects were undertaken. Initial projects were undertaken to evaluate the potencies of natural and artificial compounds in depression of methane production and their impact on feed digestion in the rumen using an artificial rumen simulation technique. Finally, an animal experiment was conducted to confirm the results obtained from the artificial rumen simulation technique.

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2. LITERATURE REVIEW

2.1. Microbiology of Methanogenesis

2.1.1. Taxonomy and diversity of methanogens

Methanogens are a distinct group of microbes that are distinguished from eubacteria by a number of characteristics, including the possession of membrane lipids composed of isoprenoids ether-linked to glycerol or other carbohydrates (Jones et al. 1987; De Rosa and Gambacorta 1988), a lack of peptidoglycan containing muramic acid (Kandler and Hippe 1977), and distinctive ribosomal RNA sequences (Balch et al. 1979; Woese 1987). This group also includes some extreme halophiles and some extremely thermophilic, sulfur-dependent microbes (Woese 1987) and is phylogenetically distinct from eukaryotes and eubacteria. Therefore, methanogens have been classified in a different domain, Archaea (formerly Archaebacteria), within the kingdom Euryarchaeota (Balch et al. 1979; Woese et al.1990; Noll 1992).

Although methanogens share many common physiological features, considerable phylogenetic diversity of methanogens is reflected in differences in the macromolecules responsible for the rigidity of the sacculus, differences in the composition of lipids, and differences in rRNA nucleotide sequences. The diversity is also reflected in the representation of cocci, various shaped rods, spirilla, thermophilic and mesophilic species, and motile and non-motile species among the different genera of methanogens.

2.1.2. Physiology of methanogens

Methanogens are fastidious anaerobes and grow only in environments with redox potentials below -300 mV (Hungate 1967; Stewart and Bryant 1988). Methanogens, however, can be "fairly tolerant" to O_2 exposure although there is a considerable variability in the sensitivity of species to O_2 (Kiener and Leisinger 1983). Most methanogens grow at neutral pH, with an optimum between 6 and 8 (Jones et al. 1987). However, some methanogen species can grow in extreme environments with pH values of 3 and 9.2 (Williams and Crawford 1985; Mathrani et al. 1988). The temperature range for methanogens is wide, with an optimum of 35°C to 40°C for mesophilic species and 60°C for thermophilic species. The maximum temperature optimum known is 98°C for one species, *Methanopyrus kandleri*.

Methanogens as a group use CH_4 formation as the sole energy-generating mechanism. The substrates used as carbon and energy sources by methanogens are restricted to a small number of simple compounds, most of which contain one carbon (Table 2.1). Many methanogens use only one or two substrates, with the greatest versatility represented in some strains of *Methanosarcina*, which can use seven substrates. Most species of methanogens can grow chemolithotrophically using CO_2 as carbon source and H_2 as energy source, and many of them can also use formate as both carbon and energy sources. Only certain species can utilize acetate, methylated amines, methylated sulfide, alcohols or CO as carbon and/or energy sources.

All methanogens use NH_4^+ as their nitrogen source and many species can fix nitrogen

Table 2.1 Substrates and major methanogenic reactions

Reaction	$\Delta G^{o'}$ (kJ/mol CH_4)	Methanogen
1. $HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O$	-135	Almost all methanogens
2. $4HCOO^- + H^+ + H_2O \rightarrow CH_4 + 3HCO_3^-$	-145	Many hydrogenotrophic methanogens
3. $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31	*Methanosarcina* and *Methanothrix*
4. $4CO + 5H_2O \rightarrow CH_4 + 3HCO_3^- + 3H^+$	-196	*Methanosarcina* and *Methanobacterium*
5. $2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$	-116	Some hydrogenotrophic methanogens[a]
6. $4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$	-105	*Methanosarcina* and other methylotrophic methanogens
7. $4(CH_3)_3\text{-}NH^+ + 9H_2O \rightarrow 9CH_4 + 3HCO_3^- + 4NH_4^+ + 3H^+$	-76	*Methanosarcina* and other methanogens[b]
8. $2(CH_3)_2\text{-}S + 3H_2O \rightarrow 3CH_4 + HCO_3^- + 2H_2S + H^+$	-49	Some methylotrophic methanogens[c]
9. $CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-113	*Methanosphaera stadtmanii*, methylotrophic methanogens

Adapted from Zinder (1993).
[a]Other short chain alcohols utilized include isopropanol.
[b]Other methylated amines utilized include mono- and di-methylamines.
[c]Methyl mercaptan (methane thiol) is also used.

when NH_4^+ is deprived (DeMoll 1993). Some species can deaminate amino acids, some hydrolyse urea, others metabolize methylamines, and some degrade purines or pyrimidines. No single methanogen has been shown to possess all of these capabilities (DeMoll 1993). Methanogens use nitrogen in the biosynthesis of amino acids, purines, pyrimidines, and other basic biochemicals by employing most of the same reactions that are used by eubacteria.

Methanogens obtain their phosphorus, as do most living organisms, in the form of

inorganic phosphate. Although phosphate metabolism in methanogens is unique in several aspects, most of the details of phosphate metabolism, such as in nucleotide synthesis, are also identical to those seen in other organisms. Methanogens use sulfide and cysteine as sources of sulfur. Methanogens also require sodium, nickel, and certain other trace elements (Mackie et al. 1992). Autotrophic species specifically require B vitamins and unknown growth factors in rumen fluid or yeast extract.

2.1.3. Ecology of methanogenesis

Methanogens cannot utilize complex organic molecules as their carbon and energy source. They are restricted to very simple and narrow range of substrates. An important consequence of this limited metabolic repertoire is that in many anaerobic ecosystems, methanogens have to consort with other organisms which metabolize complex organic matters yielding simple end-products. Therefore, conversion of complex organic matters to CH_4 requires an interacting microbial food web (consortium) (Fig. 2.1), in contrast to aerobic ecosystems, where a single organism may completely oxidize a complex organic molecule to CO_2.

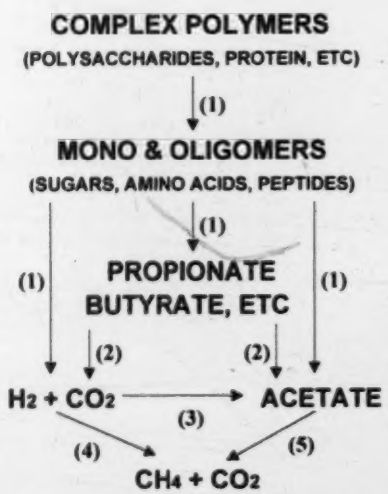

Fig. 2.1 Carbon flow in anaerobic fermentation. Microbial groups which may be involved: (1) fermentative anaerobes; (2) hydrogen-producing acetogens; (3) hydrogen-consuming acetogens; (4) hydrogen-consuming methanogens; (5) acetotrophic methanogens. (Zinder 1993).

The microbial food chain may be composed of up to four interacting metabolic groups of anaerobic microorganisms depending on the operative methanogenic habitats. No matter what the methanogenic habitats, organisms involving the first step of the degradation of complex molecules are those anaerobes including bacteria, fungi and protozoa, which hydrolyse polysaccharides and degrade the hydrolysed products to H_2, CO_2, formate, acetate, and higher fatty acids such as butyrate and propionate. These fermentative organisms also ferment protein and lipids with similar end products.

Syntrophic H_2-producing acetogens may or may not be involved in methanogenesis depending on habitat. In the anaerobic bioreactor, for example, retention time is very long (14-30 d) and fatty acids and other intermediates cannot 'escape' readily. H_2-producing acetogens thus can grow in this habitat with conversion of higher fatty acids or alcohols to acetate and H_2, and also CO_2 with odd-numbered carbon fatty acids. However, this group of organisms is rarely found in the rumen. Ruminants swallow a large quantity of water and produce large amounts of saliva so that the retention time of rumen contents is only 1-2 d. This short retention time does not allow the growth of the slow-growing syntrophic H_2-producing acetogens (doubling time > 3 d). Another reason that limits syntrophic fatty acid oxidation in the rumen is the high H_2 partial pressure of the rumen. The basal H_2 partial pressures in the rumen are about 10-fold higher than those in an anaerobic bioreactor, which is too high to allow syntrophic fatty acid oxidation (Zinder 1993). Consequently, the anaerobic food chain in the rumen is truncated and acetate, propionate, and butyrate accumulate in large amounts, with molar proportions of approximately 60 : 30 : 10.

Hydrogen-consuming acetogenic bacteria are usually found in such methanogenic

habitats as the lower gut of termites and the colon of animal. These organisms produce acetate through reduction of CO_2 with H_2. It is not clear why H_2-consuming acetogenic bacteria can outcompete methanogens in the gut of termites and the colon of human individuals. For thermodynamic reasons, the H_2-consuming acetogens are generally outcompeted by the methanogens in anaerobic bioreactors and the rumen (Zinder 1992).

Methanogens constitute the terminal group in the consortium. Sixty-six species of methanogens have been isolated from a variety of anaerobic habitats including sanitary landfills, acidic peat bogs, waterlogged soils, salt lakes, thermal environments, and the digestive tracts of animals (Archer and Harris 1986; Mackie et al. 1992). Hydrogen-consuming methanogens (hydrogenotrophs) exist in all type of anaerobic habitats, which is reflected in the fact that almost all species of methanogens can generate CH_4 using H_2 and CO_2, which are common end-products of fermentative organisms. Methanogens, rather than hydrogenotrophs, are restricted to certain habitats depending on physiological conditions and substrate availabilities. *Methanothrix*, which is often the dominant acetotrophic methanogen in anaerobic bioreactors, is rarely found in the rumen due to its slow growth rate (doubling time > 3.5 d), whereas *Methanosarcina*, with a doubling time of 1-2 d on acetate, can be found in the rumen in low numbers (Patterson and Hespell 1979). It is important in the ecosystem of ruminant that hydraulic retention time be kept short enough to prevent methanogenesis from acetate. Otherwise a significant loss of energy would occur.

Table 2.2. Characteristics of methanogens isolated from the rumen

Organism	Morphology and cell envelope composition	Nutritional requirement
Methanobrevibacter ruminantium[1]	Short rods; pseudomurein	H_2/CO_2, formate, acetate, 2-methylbutyrate, CoM[x]
Methanobrevibacter spp.[2y]	Short rods; pseudomurein	H_2/CO_2, formate, isobutyrate, isovalerate, 2-methylbutyrate, valerate, CoM
Methanosarcina barkeri[3]	Irregular cocci, large clusters; heteropolysaccharide and protein	H_2/CO_2, formate
Methanosarcina mazei[4]	Cocci; heteropolysaccharide	Methanol, methylamines, acetate
Methanobacterium formicicum[5]	Long rods and filaments; pseudomurein	H_2/CO_2, formate
Methanomicrobium mobile[6]	Short rods; protein	H_2/CO_2, formate

Adapted from [1]Smith and Hungate (1958); [2]Lovley et al. (1984) [3]Beijer (1952); [4]Mah (1980); [5]Oppermann et al. (1957); [6]Paynter and Hungate (1968).
[x]CoM, 2-mercaptoethanesulfonic acid or coenzyme M.
[y]This species includes both CoM- requiring and non- requiring strains.

2.1.4. Rumen methanogens

Six methanogen species of rumen origin have been described to date (Table 2.2). Only two species, *Methanobrevibacter ruminatium* and *Methanosarcina* isolates similar to *Methanosarcina barkeri* are assumed to play the most significant role in ruminal methanogenesis (Rowe et al. 1979; Lovley et al. 1984; Miller et al. 1986; Stewart and Bryant 1988). However, new ruminal species may be identified and the perspectives on their relative importance will no doubt change, because new species from other anaerobic habitats are frequently discovered with phylogenetic analyses of 16S rRNA (Jones et al. 1987).

Methanobrevibacter ruminantium (formerly *Methanobacterium ruminantium*), the

first methanogen isolated from the bovine rumen (Smith and Hungate 1958), is present in high numbers (10^6 - 10^8 mL^{-1}). It is a short, non-motile, Gram-positive coccobacilli. Hydrogen and CO_2 are the major energy and carbon source, respectively, for methanogenesis, although it can also use formate as energy and carbon sources, with a slower growth rate (Miller 1995).

Methanobrevibacter ruminantium requires acetate as a major source of cell carbon (Bryant et al. 1971). It is more nutritionally fastidious than other species of this genus. It requires 2-methylbutyrate, 2-mercaptoethanesulfonic acid (Coenzyme M or CoM) and a mixture of amino acids (Bryant et al. 1971; Taylor et al. 1974). 2-Methylbutyrate is a precursor of isoleucine via the Allison reductive carboxylation pathway (Robinson and Allison 1969), while CoM is a cofactor that is required for the terminal reaction of methanogenesis and found only in methanogens. The reason for the requirement of CoM by this species is not clear. CoM-requiring and non-requiring strains of *Methanobrevibacter* species have been isolated from bovine rumen contents in high numbers (10^8 - 10^9 mL^{-1}) (Lovley et al. 1984; Miller et al. 1986). Two type of strains do not react with rabbit antisera against *Methanobrevibacter ruminantium* (Miller et al. 1986). These strains require isobutyrate, isovalerate, valerate, and 2-methylbutyrate for growth.

Methanosarcina barkeri, which is often found in high numbers in anaerobic bioreactors, are also present in lower numbers (10^3 - 10^6 mL^{-1}) in the rumen of cows, goats and sheep (Beijer 1952; Rowe et al. 1979; Patterson and Hespell 1979; Vicini et al. 1987). The cells are Gram-positive, non-motile spheres, which occur in packets or large clusters. Substrates for methanogenesis include H_2/CO_2, methanol, methylamines and acetate

(Patterson and Hespell 1979; Archer and Harris 1986). The unique biochemical ability to use methanol, methylamines and acetate enables the slow-growing *Methanosarcina barkeri* to flourish in the rumen of ruminants fed diets containing ingredients like molasses that promote the utilization of methylamines, methanol and acetate as substrates for methanogenesis (Rowe et al. 1979; Vicini et al. 1987).

Methanomicrobium mobile was found in high numbers in rumen contents of cows in only one report (Paynter and Hungate 1968), and no additional information regarding its presence in the rumen has been reported. *M. mobile* grows rapidly with H_2/CO_2, but does not form CH_4 from methyl groups (Wolin and Miller 1988).

2.1.5. Biochemistry of methanogenesis

Although the sources of electrons used for the reduction of methyl group to CH_4 vary, all methanogens share a unique metabolic step in which energy conservation is associated with the reduction of a methyl group to CH_4, and coenzyme M is an essential methyl group carrier that mediates methyl group transfer to methylreductase. The central pathway is illustrated in Fig. 2.2. Six coenzymes have been confirmed as participants in the reduction of CO_2 to CH_4. These coenzymes include methanofuran (MFR), tetrahydromethanopterin (H_4MPT), cofactor F_{420}, cofactor F_{430}, coenzyme M (CoM) and 7-mercaptoheptanoylthreonine phosphate (HS-HTP). Methanofuran, tetrahydromethanopterin, and coenzyme M function as C_1-carriers, while cofactor F_{420}, cofactor F_{430} and 7-mercaptoheptanoylthreonine phosphate function as electron carriers in the reductases

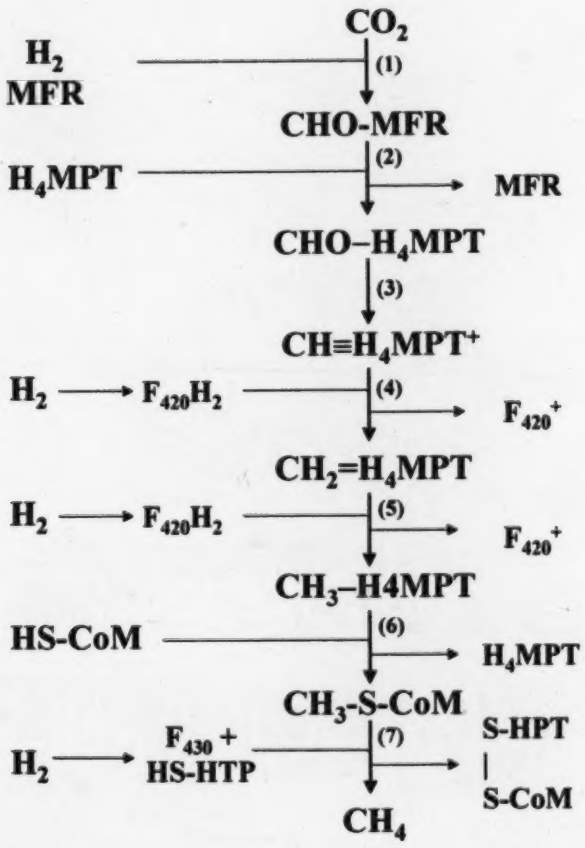

Fig. 2.2. Metabolic pathway of methanogenesis from CO_2 and H_2. MRF, methanofuran; CHO-MFR, *N*-formyl-MFR; H_4-MPT, tetrahydromethanopterin; CHO-H4MPT, N^5-formyl-H_4MPT; CH≡H4MPT$^+$, N^5, N^{10}-methenyl-H_4MPT; CH_2=H_4MPT, N^5, N^{10}-methylene-H_4MPT; CH_3-H_4MPT, N^5-methyl-H_4MPT; $F_{420}H_2$, reduced cofactor F_{420}; F_{420}^+, oxidized cofactor F_{420}; HS-CoM, coenzyme M; CH_3-S-CoM, methyl-CoM; F_{430}, cofactor F_{430}; HS-HTP, N^7-mercaptohetanoyl-*O*-phosphate-L-threonine (Thauer et al. 1993).

(DiMarco et al. 1990; Keltjens et al. 1990; Wolfe 1991).

The reduction of CO_2 to CH_4 occurs in a novel reaction in which the C_1 group is passed, bound to coenzyme, as it is sequentially reduced through the formyl, methenyl, methylene, and methyl stages to CH_4. Formyl-MFR is the first stable product of CO_2 fixation (reaction 1). The formyl group is then transferred to H_4MPT, a pterin unique to methanogens, catalysed by formyl-MFR:H_4MPT formyltransferase (reaction 2). The formyl group is next converted to a methenyl group catalysed by the enzyme 5,10-methenyl-H_4MPT cyclohydrolase (reaction 3). The reduced deazaflavin, cofactor F_{420}, donates electrons for reduction of the double bond of the methenyl group, thereby forming a methylene group in a reaction catalysed by the enzyme methylene-H_4MPT:coenzyme F_{420} oxidoreductase (reaction 4). A similar oxidoreduction reaction (reaction 5) is speculated to be involved in reduction of methylene-H_4MPT to methyl-H_4MPT (Mackie et al. 1992). Prior to reduction of methyl group to CH_4 the methyl group carried by H_4MPT is transferred to coenzyme M (reaction 6). The methyl group carried by coenzyme M, then, is reduced to CH_4 catalysed by a complex methyl-CoM methylreductase which includes cofactor F_{430} and HS-HTP (reaction 7). This terminal reaction also results in activation of CO_2 and formation of formyl-MFR.

The pathway for reduction of methanol to CH_4 has been studied in *Methanosarcina barkeri* and two methyltransferases, methanol:5-hydroxybenzinidazollyl cobamide transferase (MT1) and Co-methylcobamide:HS-CoMmethyltransferase (MT2), are involved. MT1 contains a firmly bound corrinoid and in the most reduced state [Co(I)], the corrin is methylated by methanol. The methyl group is then transferred to HS-CoM catalysed by

MT2. If methanol is used as sole substrate, part of it must be oxidized to CO_2 to provide electrons for the CH_3-CoM reductase system. This possibly occurs via a H_4MTP to form CH_3-H_4MTP and then sequentially to CHO-H_4MTP, CHO-MFR and finally CO_2. The methyl groups of trimethylamine and monomethylamine are reduced directly to CH_4 by *Methanosarcina barkeri* without exchange of protons with H_2O or the formation of free methanol as an intermediate (Mackie et al. 1992).

Aceticlastic methanogenesis involves cleavage of acetate with reduction of the methyl group (C_1) and oxidation of the carboxyl (C_2) group. Acetate is activated to acetyl-CoA, which is then cleaved by the complex enzyme system, carbon monoxide dehydrogenase, to CO_2, CoA, and a methyl moiety. The methyl group is then transferred in turn to H_4MTP and CoM before being reduced to CH_4 (Mackie et al. 1992).

2.1.6. Methanogenesis and anaerobic fermentation

2.1.6.1. Interspecies H_2 transfer

Bryant et al. (1967) found that the organism, so called "S organism", that can oxidize ethanol to acetate and H_2, grew well on ethanol only when the methanogen *Methanobacterium bryantii* was present. This finding leads to a concept of syntrophism (Greek, eating), i.e. two organisms, an H_2 producer and an H_2 consumer, could couple together break down a single substrate. The role the methanogen *M. bryantii* played in anaerobic degradation of ethanol by the "S organism" is demonstrated in the following

equations:

"S organism" $2CH_3CH_2OH + H_2O \rightarrow 2CH_3COO^- + 2H^+ + 4H_2$
$\Delta G^{o\prime} = 19.3$ kJ/rxn

M. bryantii $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$
$\Delta G^{o\prime} = -135.6$ kJ/rxn

Sum $2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$
$\Delta G^{o\prime} = -116.3$ kJ/rxn

Because the reaction in which oxidation of ethanol to acetate and H_2 is not thermodynamically favourable, the reaction will not proceed from left to right unless a product is kept at an extremely low concentration. Due to the high affinity of methanogens to H_2, the partial pressure of H_2 is kept as low as 10^{-4} atm in the presence of these organisms (Hungate 1975). This is low enough to result in the formation of H_2 from NADH + H^+ (Gottschalk and Andreesen 1979). NADH has a redox potential of -320 mV that is not low enough to produce H_2 (E'_o= -414 mV at pH 7) under standard conditions. However, if the H_2 partial pressure is kept as lower than 10^{-3} atm (ca. -320 mV), then H_2 production from NADH becomes feasible. Microbial associations in which a H_2-producing organism can grow only in the presence of a H_2-consuming organism are termed obligate syntrophism. The coupling of formation and utilization of H_2 is called interspecies H_2 transfer. Other similar associations have been discovered, including those responsible for oxidation of acetate, butyrate, propionate, and benzoate.

However, many anaerobes do not require interspecies H_2 transfer to ferment organic matter, although they benefit from it. Many rumen carbohydrate-utilizing microorganisms, including bacteria and fungi, are good examples. *Ruminococcus albus* can produce H_2 from pyruvate oxidation to acetyl-CoA and CO_2 (Miller and Wolin 1973). This reaction is not

inhibited by H_2 because the enzyme pyruvate:ferredoxin oxidoreductase involved has an oxidation reduction potential near -400 mV, close to that of H_2. In contrast, the organisms can also produce H_2 from oxidation of NADH (E'_o= -320 mV) by the action of NADH:ferredoxin oxidoreductase and ferredoxin hydrogenase, but this reaction is thermodynamically unfavourable unless the H_2 partial pressure in the system is low (Glass et al. 1977). When H_2 accumulates, the formation of H_2 from NADH is inhibited and NADH produced during glycolysis is oxidized through the alternative pathway of ethanol formation catalysed by the enzyme alcohol dehydrogenase, where NADH functions as an electron donor. When H_2 is removed by methanogens or other H_2-consuming organisms, H_2 is produced from NADH. This allows the organism to dispose of electrons from NADH as H_2 rather than having to dispose of them by producing more reduced products such as ethanol (Fig. 2.3). The net benefit for *R. albus* from interspecies H_2 transfer is, therefore, one more ATP conservation.

The fermentations of several other important microorganisms in the rumen are also influenced by the partial pressure of H_2 and subject to alteration of biochemical pathways by interspecies H_2 transfer. *Ruminococcus flavefaciens*, another cellulolytic bacterium, can oxidize NADH by reduction of oxaloacetate through a series of reactions to succinate when it is cultured alone. When it is co-cultured with a methanogen, succinate production by *R. flavefaciens* is dramatically decreased and acetate production is increased (Latham and Wolin 1977; Stewart et al. 1990). Similarly, when *Selenomonas ruminantium* is co-cultured with a methanogen, carbon flow turns to acetate formation at the expense of propionate (Chen and Wolin 1977). Figure 2.4 illustrates the diversion of carbon flow from succinate to acetate

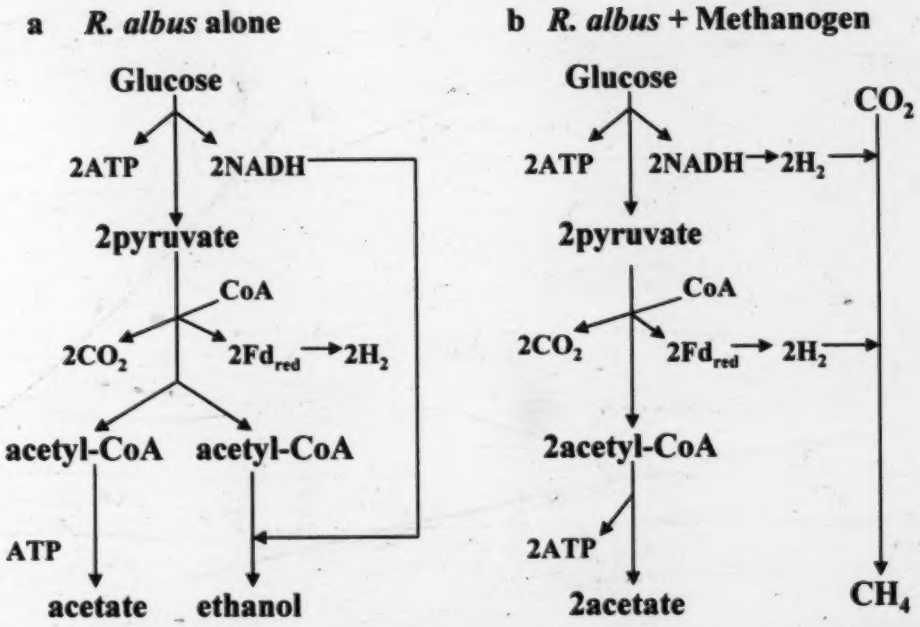

Fig. 2.3 Fermentation products of *Ruminococcus albus* growing axenically on cellulose (a) or with a methanogen (b) (Miller and Wolin 1973).

by the influence of interspecies H_2 transfer.

In addition to fermentative bacteria, the most obvious beneficial effect of methanogens on non-methanogens occurs with H_2- or formate-producing anaerobic fungi (Bauchop and Mountfort 1981; Marvin-Sikkema et al. 1990; Stewart and Richardson 1990). Lactate and ethanol, major end-products of rumen fungi found in an axenic culture, are decreased, while acetate production is increased when rumen fungi grow with a methanogen (Fig. 2.5). It was found that when rumen fungus *Neocallimastix frontalis* was co-cultured with a methanogen, the production of lactate dehydrogenase, acetaldehyde dehydrogenase, alcohol dehydrogenase and fumarate reductase, which are key enzymes for synthesis of lactate, ethanol and succinate, were depressed (Marvin-Sikkema et al. 1993).

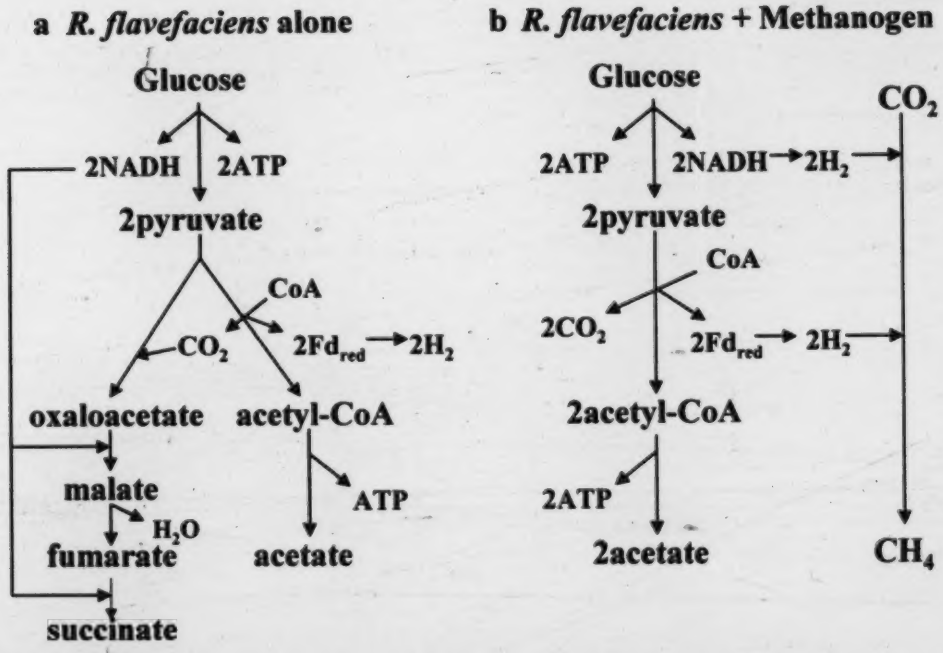

Fig. 2.4 Fermentation products of *Ruminococcus flavefaciens* growing axenically on cellulose (a) or with a methanogen (b) (Latham and Wolin 1977).

Several species of flagellate, ciliate and ameboid protozoa have been commonly found in anaerobic sediments and animal gastrointestinal tracts. These anaerobic protozoa lack mitochondria, but often contain membrane bound organelles, called hydrogenosomes, in which pyruvate is converted to acetyl-CoA, CO_2, and H_2 (Müller 1988).

It has first been noted that methanogens attach on the surfaces of rumen ciliate protozoa based on autofluorescence of F_{420}-containing methanogen cells (Vogels et al. 1980). Since that time methanogens, either attaching to the surface or living inside anaerobic protozoa, have been found in the termite guts, cockroach guts and aquatic sediments (van Bruggen et al. 1986; Lee et al. 1987; Finlay and Fenchel 1991; Guzen et al. 1991).

It has been demonstrated that the density of methanogens attached on the surfaces of

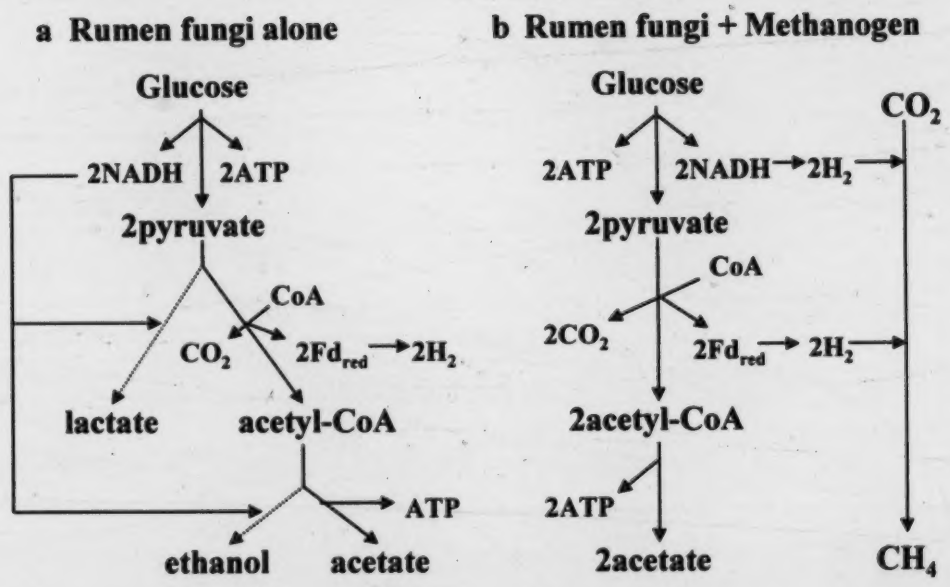

Fig. 2.5 Fermentation products of rumen fungi growing axenically on cellulose (a) or with a methanogen (b) (Marvin-Sikkema et al. 1990).

rumen protozoa is reduced when exogenous H_2 is supplied (Stumm et al. 1982; Smolenski and Robinson 1988), and it is reasonable to assume that the role of the symbiotic relationship between methanogens and anaerobic protozoa resembles facultative interspecies H_2 transfer, thereby allowing the protozoa to dispose of their electrons as H_2 and produce more acetate. Hino (1983) reported that starch fermentation and ATP synthesis by *Entodinium* spp. were reduced under a high partial pressure of H_2. The inhibition disappeared when *Entodinium* spp. were co-cultured with rumen methanogens. Stumm et al. (1982) estimated that 10-20% of methanogens are attached on the surfaces of protozoa in the rumen. Even though the relationship between methanogens and protozoa is not clear (Krumholtz et al. 1983), the study conducted by Holler and Pfennig (1991) demonstrated that the anaerobic ciliate

Trymyema compressum infected by the methanogen *Methanobacterium formicicum* did not increase acetate production and host cell yield as compared to protozoa without methanogens. Little or no effect was found on host cell yield in a similar study of three anaerobic ciliates with methanogens eliminated using BES (Fenchel and Finlay 1991), although fermentation products were not measured in this study. Other studies have also indicated that protozoa did not produce more H_2 in the presence of the methanogen than in the absence of the methanogen (Zinder 1993). It has been assumed that anaerobic protozoa may not possess the biochemical mechanism to convert NADH to H_2 (Zinder 1993). Nevertheless, the experimental results consistently demonstrate that defaunation reduces methanogenesis in the rumen (Whitelaw et al. 1984; Itabashi et al. 1984; Ushida et al. 1986; Vermorel and Jouany 1989).

2.1.6.2. Interspecies formate transfer

A hypothesis was put forward that formate rather than H_2 may function as the mediator carrying electrons from one organism to the other, i.e. interspecies formate transfer occurred. Such secretion and uptake of formate could be electrogenic, thereby contributing to the proton motive force in both syntrophic partners (Thiele and Zeikus 1988; Boone et al. 1989). The experimental data supporting this hypothesis was that *Oxalobacter formigens* conserved energy from formate excretion (Allison et al. 1985), and *Methanobacterium formicicum* showed 1.4-fold higher growth efficiency (Y_{CH4}) in its presence (Schauer and Ferry 1980). In addition, Boone et al. (1989) examined a methanogenic butyrate-oxidizing

co-culture and found that the dissolved H_2 concentrations were near 63 nM during active metabolism, dropping to a minimum threshold of 35 nM near the end of growth. A formate concentration of 16.4 μM was thermodynamically equivalent to 63 nM H_2. Using a diffusion model with these concentrations, it was calculated that even though the diffusion coefficient of formate is five-fold lower than that of H_2, formate could be expected to transfer electrons 98-fold more rapidly than H_2.

However, a clear argument against the necessity of interspecies formate transfer is that syntrophic ethanol and butyrate-degrading cultures exist which grow in the presence of methanogens that are unable to oxidize formate (Bryant et al. 1967; McInerney et al. 1981; Ahring and Westermann 1987). In propionate-adapted methanogenic granular sludge, interspecies formate transfer is not important because in thin sections of the sludge propionate oxidizers were found to be surrounded by *Methanobrevibacter arboriphilus*, a methanogen that cannot use formate (Zehnder and Wuhrmann 1977).

Many rumen microorganisms produce formate and H_2 when they are grown axenically, but no formate is detectable in the rumen (Hungate et al. 1970). Thus, an interspecies formate transfer may play a role in electron transfer in the rumen habitat. However, its biological significance has not yet been fully established, although it has been estimated that formate accounts for 18% of rumen methanogenic substrate (Hungate et al. 1970; Whiteman et al. 1992). However, formate-hydrogen lyase enzyme systems are common in anaerobes and formate and H_2 can be interconverted according to the following equation:

$$HCOO^- + H_2O \leftrightarrows HCO_3^- + H_2$$

Thus, formate/H_2 equilibrium would be expected in most anaerobic habitats, and determination of the relative roles of formate versus H_2 in interspecies electron transfer needs to be assessed under a variety of conditions, including pH, temperature, and bicarbonate concentrations because these factors can affect the H_2/formate equilibrium.

2.1.6.3. Summary

The net effect of interspecies H_2 and/or formate transfer in which methanogens are involved with other anaerobes is that there is a reduction in the amount of the pyruvate which must be reduced for NAD regeneration, instead pyruvate is oxidized to acetyl-CoA, and the high-energy thioester bond in this compound can be conserved as ATP via acetyl phosphate. For example, an organism carrying out glycolysis of sugars and producing lactate only conserves two ATP equivalents from glucose. However, if two mole of acetate are formed instead, the net equivalent of four ATP molecules are conserved, benefiting the H_2-producing organisms. Thus, the presence of H_2- and/or formate-consuming methanogens often causes in many fermentative anaerobes, such as rumen fibrolytic bacteria and fungi, to switch their metabolism toward the pathway with a greater ATP conservation, consequently leading to a higher cell yield of fermentative microorganisms and an enhanced extent of organic matter fermentation. In contrast, if methanogens are absent, a lowered extent of organic matter fermentation and total microbial yield would be expected.

2.2. Inhibition of Ruminal Methanogenesis

2.2.1. Manipulation of ration and feeding strategy

Methane production in the rumen can be moderated by a diet rich in rapid fermentable carbohydrate such as starch (Ørskov et al. 1968; Demeyer and Van Nevel 1975). Lowered CH_4, associated with increased propionate and decreased acetate, may be attributed to a rapid ruminal fermentation, which favours propionate-producing microorganisms such as *Selenomonas ruminatium* over methanogens (Demeyer and Van Nevel 1975). In addition, rapid ruminal fermentation of starch or soluble carbohydrates often results in pH less than 6.0, which may further inhibit growth of methanogens and/or other H_2- or formate-producing microorganisms, including fibrolytic bacteria and rumen protozoa, which are usually favoured in neutrality of pH (Demeyer and Henderickx 1967; Eadie et al. 1970; Van Kessel and Russell 1995).

Methane production of ruminants is generally increased when the proportion of dietary roughage is increased in their ration, but varies depending on digestibility of fibre sources, physiological maturity, method of preservation, chemical and physical treatment. With highly digestible fibre sources like beet pulp, CH_4 production may be lowered to 4-5% of gross energy intake (Kujawa 1994). CH_4 production is generally lower when ruminants are fed on legume than grass (Varga et al. 1985), and tends to increase with the maturity of forage fed (Armstrong 1960). Ensiling, grinding and pelleting lowers CH_4 production (Sundstøl 1981; Thomson 1972; Hironaka et al. 1996). Chemical treatment of cereal straws

with NaOH or ammonia decreases CH_4 production per unit of digestible organic matter (Moss et al. 1994), but the intensity of depression varies with the straw type. Maximum depression is obtained with wheat straw treated with ammonia.

Methane production in ruminants decreases with increasing level of feed intake. The amount of energy lost as CH_4 per unit of feed intake decreases to 12% from 30% when feed intake is increased from maintenance to twice maintenance, although total CH_4 production increases (Blaxter 1967). A comparison of 42 forages indicated that CH_4 production decreased by about 7% as intake of forages was increased by about 40% (Mathison, unpublished data). He also found that CH_4 production was depressed by 42% as intake of concentrate diets increased by 71%. A much greater extent of depression in CH_4 production with increasing intake level of concentrate diets may be partly attributed to defaunation of rumen protozoa because concentrate diets fed at maintenance level causes a proliferation in the ciliate population (Bonhomme 1990), and increasing intake level reduces protozoa population and CH_4 production (Dearth et al. 1974; Kreuzer et al. 1986).

Increasing level of feed intake may affect not only the population of rumen protozoa, but also the extent of substrate availability to microorganisms in the rumen. Generally, high intake results in high passage rate of feed particles out the rumen (Owens and Goetsch 1986). Consequently, the extent to which microbes access to substrates is decreased which, in turn, reduces the rate and/or extent of ruminal dietary fermentation and thus CH_4 production. However, the extent to which intake level affects passage rate of roughage is proportionately less than with concentrate or mixed diets. When mixed diets with less than 25% concentrate are fed, the effect of intake level on passage rate of roughage is limited (Galyean and Owens

1991). This supports the suggestion that intake level has less effect on CH_4 production in ruminants fed roughage-based diets than concentrate-based diet.

Feeding strategy also affects ruminal CH_4 production. Low frequencies of feeding tend to increase propionate production and lower CH_4 production (Roth and Kirchgessner, 1976; Jensen and Wolstrup 1977; Sutton et al. 1986). Again, this effect is probably associated with lowering the population of rumen protozoa. Moir and Somers (1956) found that feeding the same quantity of feed four times a day instead of once daily resulted in a doubling population of protozoa, whereas the total bacterial population was not affected. The most plausible explanation for this may be that multiple feedings prevent the drastic fluctuations in rumen pH which can be inhibitory to protozoa. The experiment conducted by Kaufmann et al. (1980), for instance, demonstrated that when a given level of concentrate diets was fed to cows twice a day, rumen pH fluctuated from 5.85 to 6.65, whereas when cows were fed six times a day, rumen pH ranged only from 6.15 to 6.4. Bragg et al. (1986) determined the diurnal pattern of rumen pH and protozoa population of steers fed on a corn silage-concentrate (40:60) diet either twice or eight times daily, and found that populations of protozoa were slightly higher when the steers were fed eight times a day than twice a day, with minimum pH values being 5.8 and 5.45, respectively.

In summary, reduced CH_4 production in the rumen is possible by manipulating ration composition and feeding strategy. A reduction in methane production is associated with lowered population of rumen protozoa, which are symbiotically related to methanogens. Therefore, it appears that removal of protozoa from the rumen would be important in terms of reducing ruminal CH_4 production.

2.2.2. Defaunation

Defaunation is a technical term for eliminating protozoa from the rumen. It was found that defaunation resulted in reduction of ruminal CH_4 production by 20 to 50% in ruminants (Kreuzer et al. 1986; Williams and Coleman 1992). The huge variations observed were related to differences in diet composition (see above). The effect of defaunation on ruminal methanogenesis can be attributed to several factors, such as the lower rumen digestion of fibre (Veira et al. 1983), the loss of methanogens that are normally symbiotically related with protozoa (Krumholz et al. 1983), and the loss of H_2 and/or formate-producers for methanogenesis (Müller 1988). In addition, Van Nevel and Demeyer (1996) also suggest that a shift in the site of digestion from rumen to hindgut, which is associated with defaunation, reduces ruminal methanogenesis. However, some literatures reviewed by Van Nevel and Demeyer (1988) indicate that defaunation increases rumen volume, thereby increasing mean retention time of digesta in the rumen, which would compensate the negative effect on ruminal digestion of diet, especially on fibre digestion, caused by loss of protozoa.

Unfortunately, at present, there are no satisfactory defaunation methods available to apply on a practical scale. Methods used in experimental conditions can cause serious weight loss of animal due to severe anorexia during treatment and even death (Kreuzer et al. 1986; Jouany et al. 1988). Therefore, more research in developing defaunation methods is needed.

2.2.3. Channelling of electron flow

Methanogens carry out important terminal reactions in which remove the end-products produced by fermentative microorganisms. The consequence is the shift in fermentation pattern to more energy yield. On the other hand, methanogens also compete for substrates with other organisms, especially H_2-utilizing organisms such as nitrate, sulfate and iron reducers as well as H_2-consuming acetogens. Because the magnitude of free energies of many anaerobic reactions is small, concentration of products and reactants can have a major effect on whether or not the reaction is thermodynamically favourable. The population of organisms in the habitat is determined by the amount of energy generated during substrate oxidation.

Most *Desulfovibrio* species grow on H_2 as an electron donor and $SO_4^{=}$, $SO_3^{=}$ or $SSO_3^{=}$ as electron acceptors (Badziong et at. 1978). Sulfate reducers are capable of using H_2 at lower partial pressure than methanogens and steady-state H_2 levels are usually very low in habitats receiving modest amounts of organic matter. Thus, sulfate reducers generally outcompete methanogens. However, some *Desulfovibrio* species can produce H_2 when growing fermentatively on pyruvate (Postgate 1952) and lactate (Bryant et al., 1977) in the absence of sulfate. Pyruvate is oxidized to acetate, CO_2, and H_2. This process is thermodynamically favourable ($\Delta G^{o'}$ = -189 kJ/reaction), whereas degradation of lactate is not thermodynamically favourable ($\Delta G^{o'}$ = -8 kJ/reaction). The fermentation can proceed only when it is coupled with the reaction associating with H_2-consuming methanogens or other bacteria. When growing on lactate by interspecies H_2 transfer with methanogens,

Desulfovibrio species can be purely fermentative organisms deriving their energy from substrate-level phosphorylation.

Dissimilatory sulfate reduction is a transmembrane redox process with external oxidation of H_2 and vectorial electron transport. The electron from H_2 is transferred to the hydrogenase located in the periplasmic side of cytoplasmic membrane in close association with cytochrome c_3 (Badziong and Thauer 1980). Due to the spatial arrangement of the electron transport components in the membrane, when H_2 is oxidized, the protons (H^+) remain outside of the membrane, whereas the electrons are transferred across the membrane. In this way, a proton motive force is established to generate ATP. In the cytoplasm, the electrons are used to reduce adenosine phosphosulfate (APS), the activated form of sulfate. In dissimilatory sulfate reduction, the sulfate ion of APS is reduced directly to sulfite. Once sulfite is formed, the subsequent reduction proceeds readily by many organisms using it either as an electron acceptor or in detoxification of sulfite (Brock and Madigan 1991). The sulfite is reduced to hydrogen sulfide by sulfite reductase.

Ability to utilize NO_3^- and/or NO_2^- as terminal electron acceptors is widespread among anaerobes. Since the amount of energy available to nitrate-reducing bacteria is so much greater than methanogens and sulfate reducers, nitrate reducers can easily outcompete these other anaerobes.

The biochemistry of nitrate and nitrite reduction has been studied in most detail in *Escherichia coli* that uses NO_3^- as a terminal electron acceptor when O_2 is absent by virtue of a dissimilatory nitrate reductase which is induced when nitrate is provided (Ingledew and Poole 1984). NO_3^- is very rapidly reduced to NO_2^-. The reaction

$$NO_3^- + 2e^- + 2H^+ \rightleftharpoons NO_2^- + H_2O$$

has an oxidation-reduction potential of +420 mV at pH 7, giving a $\Delta G^{o'}$ for nitrate reduction by NADH of -163 kJ/mol, a large enough standard free energy for the reaction to be coupled to the synthesis of more than 1 ATP/2e^-. The utilization of nitrate as terminal oxidant does not incur the metabolic consequences that accompany use of fumarate so the carbon source may be fully oxidized and, assuming similar stochiometries for oxidative phosphorylation with O_2 (Ingledew and Poole 1984). The ATP yields will be the same as with O_2.

In the absence of O_2, *E. coli* can also rapidly reduce NO_2^- to NH_4^+ (Ingledew and Poole 1984). At least three separate pathways contribute to this process: a soluble NADPH:sulfite reductase, which also has a measurable activity with nitrite; a soluble NADH:nitrite reductase; and a respiratory chain-linked (partially membrane-dependent) nitrite reductase. The reduction of nitrite to ammonia is a six-electron reduction:

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O$$

In the case of the NADPH:sulfite and NADH:nitrite reductase, the whole process is catalysed by one enzyme. It is not known whether the entire reduction of nitrite is catalysed by a single terminal enzyme in the case of the respiratory chain system, but the terminal enzyme is a periplasmic cytochrome c_{552} that can reduce nitrite to ammonia. The overall oxidation-reduction potential for the six-electron transfer reaction is +275 mV, a value between those of fumarate and nitrate couples and enough to support oxidative phosphorylation and give a $\Delta G^{o'}$ for NADH oxidation of -113 kJ/mol. Although the product of these pathways is NH_4^+, the control of the processes is such that they do not appear to have an assimilatory function, but rather the removal of catabolic reducing equivalents (Ingledew and Poole

1984). The nitrite reductases are induced by nitrite and repressed by O_2 and nitrate (Abou-Jaoude et al. 1979). Ammonia has no effect on nitrite reductases (Cole et al. 1974; Jackson et al. 1981).

In the rumen, NO_3^- is rapidly reduced to NO_2^-. Nitrite can be subsequently reduced to NH_4^+, but at much slower rate (Wang et al. 1961). When NO_3^- is present in sufficient quantities such that the rate of its reduction exceeds the capacity for NO_2^- reduction, accumulated NO_2^- will diffuse into the bloodstream. Nitrite oxidizes haemoglobin to methaemoglobin which cannot transport O_2 and also acts as a vasoconstrictor.

E. coli as well as a few eucaryotic microorganisms can grow anaerobically using fumarate as an electron acceptor via the fumarate reductase system (Ingledew and Poole 1984; Gottschalk 1986). Fumarate serves as an oxidant for anaerobic respiration by being the oxidized half of the succinate-fumarate couple, which has an oxidation-reduction potential of +30 mV, giving a $\Delta G^{o'}$ for fumarate reduction to succinate by NADH of -67 kJ/mol, which is large enough to be coupled to proton translocation and ATP synthesis. In addition to NADH, H_2, and formate can donate electrons to fumarate reductase and α-glycerol phosphate *(E. coli)* and lactate (most propionic acid bacteria) have been shown to function as NAD^+-independent donors for fumarate reductase.

In *E. coli*, the succinate-fumarate couple may be utilized as either oxidant or reductant for the respiratory chain. These two reactions are catalysed by two different enzymes, succinate dehydrogenase and fumarate reductase. Although these two enzymes are strikingly similar in structure, the genetic independence of the two enzymes was suggested by variation of the two activities in a nonparallel manner (Hirsch et al. 1963). Thus, succinate

dehydrogenase is induced aerobically and repressed anaerobically, whereas fumarate reductase is repressed in the presence of O_2 or NO_3^- but expressed anaerobically and in the presence of fumarate (Hirsch et al. 1963; Spencer and Guest 1973). Although fumarate reductase had both succinate-oxidizing and fumarate-reducing activities under appropriate conditions in vitro, the K_m and V_{max} values of the enzymes for the two substrates were substantially different in dye-linked assays. The fumarate reductase had a K_m of 17 μM for fumarate, whereas it was 1,000 μM for succinate. The succinate dehydrogenase had K_m of 260 and 450 μM for succinate and fumarate, respectively (Ingledew and Poole 1984).

Unsaturated fatty acids were found to inhibit methane production in the rumen (Czerkawski et al. 1966a; 1966b; Demeyer and Henderickx 1967; Demeyer et al. 1969). It was thought to be attributed to competition with methanogens for H_2 that is alternatively used to hydrogenate unsaturated fatty acids (Lennarz 1966). Hydrogenation of unsaturated fatty acids such as linoleic and linolenic acid occurs in the rumen and this process is not confined to lipolytic bacteria (Kemp et al. 1975; Hazlewood et al. 1976).

Hydrogenation of unsaturated fatty acids absolutely requires a free carboxyl group. Thus, lipids must firstly be unesterified (Kepler et al. 1970; Hazlewood et al. 1976). The initial step of hydrogenation is isomerization resulting in the formation of a conjugated *cis*-9,*trans*-11 acid. Subsequently, hydrogenation of the conjugated diene takes place in the transfer of 2 H to each double bond in turn, the *trans*-11 being the last to be hydrogenated (Harfoot and Hazlewood 1988). Hydrogenation of the conjugated acid is catalysed by *cis*-9,*trans*-11 octadecadienoate reductase (Hughes et al. 1982) and electron transfer from NADH is supposedly mediated by a flavin-like and a quinone-like substance (Yamazaki and

Tove 1979; Hughes and Tove 1980a; 1980b).

2.2.4. Chemical inhibitors of methanogenesis

Inhibition of methanogenesis by variety of chemicals has long been studied. Chlorinated methane analogues, such as chloroform, carbon tetrachloride, and methylene chloride were first reported by Bauchop (1967) to inhibit methanogenesis in rumen samples. Subsequently, Prins et al. (1972) demonstrated inhibition of pure cultures by micromolar levels of chloroform and carbon tetrachloride. Chloroform significantly inhibited methanogenesis. The mechanism is inhibition of methyl-CoM reductase (Gunsalus and Wolfe 1978). However, halogenated alkanes are known to block the functioning of corrinoid enzymes in methanogenesis (Wood et al. 1968; Kenealy and Zeikus 1981). These compounds can interfere with the functioning of B_{12} enzymes (Oremland and Capone 1988). Therefore, their specificity for the "target" organism is debatable. Furthermore, chloroform, carbon tetrachloride, dichloromethane, as well as chlorinated ethanes and ethenes are all susceptible to anaerobic degradation (Rittman and McCarty, 1980; Bouwer and McCarty 1983a).

α-Bromoethanesulfonic acid has been studied as a more potent inhibitor of methanogenesis. BES is a structural analogue of mercaptoethanesulfonic acid, the co-factor known as coenzyme M in methanogens. BES was first employed to study the inhibition of methyl-CoM reductase activity in cell-free extracts of *Methanobacterium thermoautotrophicum* (Gunsalus et al. 1978). Fifty percent inhibition of methane production

occurred at approximately 10^{-5} M BES, with total inhibition evident at approximately 5×10^{4} M. In contrast, chloroethanesulfonic acid required about 10-fold higher concentrations than BES to achieve the same degree of inhibition. In addition, BES was found to be a potent inhibitor of methanogenesis and growth by *Methanobacterium ruminantium*, a rumen methanogen that requires an exogenous supply of CoM (Taylor et al. 1974), although there was some ameliorating influence related to the amount of HS-CoM in the medium. At a molar ratio of 15:1 (HS-CoM:BES) no inhibition could be achieved, whereas complete inhibition occurred at a ratio of 1:1. By contrast, an analogue of BES, β-bromopropanesulfonic acid (BPS), was ineffective as an inhibitor (Balch and Wolfe 1979a). Uptake of HS-CoM by whole cells of *M. ruminantium* was completely inhibited by 10^{-6} M BES, whereas 10^{-5} M BPS had no inhibitory effect (Balch and Wolfe 1979b). Another promising candidate as an inhibitor of methyl-CoM reductase is seleno-CoM (Oremland and Capone 1988). However, only very limited work has been done with this compound.

α-Bromoethanesulfonic acid inhibits methanogenesis by all species of methanogens growing on any of the recognized substrates (Oremland and Capone 1988). Since CoM is found only in methanogenic bacteria, BES functions as a specific inhibitor when used in mixed microbial systems. In addition, BES is inexpensive and readily obtained from commercial sources in a purified form. Although it functions as a competitive inhibitor, relatively little should be required because the quantities of CoM in the environment are in the nanomolar range (Mopper and Taylor 1986). α-Bromoethanesulfonic acid is also water soluble. Thus, it can easily be added into the rumen and other habitats, such as sewage sludge, and sediment slurries. The compound is usually effective at blocking

methanogenesis in cultures when added at levels in excess of 0.1 mM (Oremland and Capone 1988).

α-Bromoethanesulfonic acid, however, does have some special problems related to spontaneous mutants which are resistant to BES. Spontaneous mutants of *Methanosarcina* strain 227, for example, were resistant to 0.24 mM BES when previously exposed to levels of 0.024 mM, and a strain of *Methanobacterium formicicum* was found to be resistant to 0.2 mM BES without any prior exposure (Smith and Mah 1981). Resistance to BES appears to be conferred by impermeability to the compound, since cell-free extracts of resistant mutants were susceptible to BES inhibition of methyl-CoM reductase (Smith 1983). Bouwer and McCarty (1983b) reported that fixed film digesters had only partial (41%) inhibition when exposed to 0.6 mM BES. They speculated that this may have been due to resistant mutants or degradation of BES by the microbial community.

A considerable amount of research has been done on the influence of the ionophores on methanogenesis. Monensin is one of ionophores studied most extensively and used commercially. In addition, others, such as lasalocid, tetronasin, lysocellin, narasin, and laidlomycin, either have been investigated or are used commercially. Monensin addition to populations of mixed rumen bacteria inhibited methanogenesis and increased propionate formation (van Nevel and Demeyer 1977). However, when hydrogen gas was added to the mixed cultures containing monensin, CH_4 production was restored, and even increased, suggesting that monensin does not directly act on methanogens. Subsequent studies revealed that monensin directly inhibited growth of some methanogens, namely *Methanobacterium bryantii, Methanobacterium formicicum,* and *Methanosarcina barkeri*, whereas,

Methanobacterium ruminantium, a ruminal methanogen, was not inhibited (Chen and Wolin 1979). Similarly, cellulolytic ruminal bacteria, *Ruminococcus albus* and *R. flavefaciens* as well as *Butyrivibrio fibrisolvens*, were particularly sensitive to monensin, but *Bacteroides succinogens* was eventually able to grow in the presence of 2.5 μg of monensin. The sensitivities of some cellulolytic bacteria and the long lag time of *B. succinogenes* may explain why short-term in vitro experiments have often shown depressed cellulose digestion, while in vivo feeding trials have exhibited no depression.

Ruminal protozoa were inhibited by monensin in vitro (Hino 1981), but not all workers have observed a decrease in protozoan numbers in vivo (Eadie and Gill 1971). Because protozoa produce H_2 and are colonized by methanogens, their elimination would contribute to reductions in ruminal CH_4 production. In vitro experiments showed that ruminal fungi were also sensitive to monensin (Stewart et al. 1987). However, in vivo inhibition has not been confirmed. Ruminal fungi produce large amount of H_2 (Wallace and Joblin 1985). There has been considerable speculation on the importance of ruminal fungi in the degradation of cellulose, and in particular highly lignified cellulose (Mountfort 1987). However, it has estimated that the ruminal fungi account for only 8% of the total ruminal biomass (Citron et al. 1987).

Ionophores are highly lipophilic substances which are able to shield and delocalize the charge of ions and facilitate their movement across membranes. Some ionophores function as mobile carriers within the membrane. Mobile carriers are selective for specific ions. Others, commonly called pore formers, create channels which traverse the cell membrane and have less specificity. Monensin is an antiporter with a high selectivity for

Na^+, but it also has the ability to translocate K^+. Thus, monensin is able to collapse Na^+ and H^+ gradients existing across biological membranes.

Ionophores are antibiotics (Pressman 1976). Thus, resistance of bacteria to ionophores will cause concern. Yet after more than 10 years of widespread use, ionophores continue to improve the efficiency of animal performance (Russell and Strobel 1989). This observation would suggest the sensitivity of ruminal microorganisms to ionophores is relatively stable and that the pattern of resistance is due to a fundamental difference between cells.

2.3. Conclusions

Methanogenic bacteria carry out important terminal reactions in anaerobic fermentation. They interact mutualistically with fermentative microorganisms in the rumen and other anaerobic habitats by removing H_2, thereby shifting the flux of carbon towards the degradation pathway. In the absence of methanogens, anaerobic fermentation may not proceed normally. This phenomenon is true for some syntrophic anaerobes which obligately rely on interspecies H_2 transfer for fermentation of organic acids. However, many, if not all, fermentative microorganisms in the rumen, including bacteria and fungi, possess biochemical mechanisms for regeneration of NAD through alternative reductive pathways using NADH. These anaerobes do not necessarily require methanogens for electron disposal. Therefore, removal of methanogens from the rumen may not seriously depress ruminal fermentation of feedstuffs. Additionally, removal of methanogens from the rumen may cause a shift of

acetate production to more reduced products, such as ethanol, lactate, succinate and propionate. Lactate and propionate are important precursors of gluconeogenesis in ruminant animals. Therefore, depression of ruminal methanogenesis would benefit ruminant animals with an improved efficiency of diet energy utilization.

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3. LIPID-INDUCED DEPRESSION OF METHANE PRODUCTION AND DIGESTIBILITY IN THE ARTIFICIAL RUMEN SYSTEM (RUSITEC)[1]

3.1. Introduction

Inhibition of methane production in the rumen by long-chain unsaturated fatty acids has been recognized for many years (Czerkawski et al. 1966a). Depression of methane production increases with the degree of fatty acid unsaturation. However, the reduction in methane production induced by unsaturated fatty acids is not directly proportional to the number of double bonds introduced. For example, reduction in methanogenesis by linolenic acid is considerably greater than would be indicated by stoichiometric calculation, even based on hydrogenation of all three double bonds (Czerkawski et al. 1966b). Thus, factors other than consumption of hydrogen via biohydrogenation are involved. A direct toxic effect of unsaturated fatty acids on methanogens has also been reported for mixed rumen bacteria in vitro (Demeyer et al. 1967). In pure culture, growth of *Methanobacterium ruminantium* is severely inhibited by long chain fatty acids with the order of inhibitory activity of a series of long chain fatty acids being: $C_{18:1}>C_{14}>C_{12}>C_{16}>C_{18}$ (Prins et al. 1972; Henderson 1973). The inhibitory pattern of long-chain fatty acids was similar for Gram-positive bacteria, but Gram-negative bacteria were less sensitive to fatty acids at the same concentration (Galbraith et al. 1971; Maczulak et al. 1981).

Lipids of either plant or animal origin depress fibre degradation both in the rumen and in the total digestive tract (Doreau et al. 1991; Ferlay and Doreau 1992), but the extent

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of this depression varies with the nature and amount of lipids used, the animal species and the experimental conditions. In contrast, lipid supplementation often does not affect the digestion of starch or soluble carbohydrates in ruminants (Tamminga et al. 1983).

Canola oil, which contains predominantly oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids (Weiss 1983), is sometimes used as a high energy supplement to finishing diets of feedlot cattle in western Canada. Engstrom et al. (1994) found that the inclusion of 4% canola oil in a typical finishing diet tended to increase the average daily gain, but did not improve feed efficiency. The direct incorporation of fatty acids into animal lipids (Nehring et al. 1963; Czerkawski et al. 1966a) and a reduction in CH_4 production would be expected to improve efficiency of metabolizable energy (ME) utilization. However, these positive effects may be counteracted by a reduction in DM digestibility.

The present study was undertaken to compare canola oil, cod liver oil (also predominately unsaturated fatty acids but with a different fatty acid profile), and coconut oil (predominantely saturated with more toxic C_{12} and C_{14} fatty acids) on feed digestibility, microbial populations and methane production. A hay diet (fibrous) and a wheat-based diet (very rapidly degraded starch) were used to provide a range in dietary conditions. The experiment was conducted using the rumen simulation technique (RUSITEC).

3.2. Materials and Methods

3.2.1. Diet preparation

Early bloom orchardgrass (*Dactylis glomerata*) hay and wheat (*Triticum* sp.) grain were used to prepare two basal diets consisting of 100% hay (hay diet) or a mixture of 90% wheat and 10% hay (concentrate diet). Hay and wheat were ground through a 3-mm screen. The ground wheat was then sieved and particles passing through a 2.0-mm screen, but retained by a 0.85-mm screen were collected. Experimental diets were prepared by spraying the basal diets with 10% (wt wt^{-1}) canola oil (Flora Distributors Ltd., Vancouver, BC), cod liver oil (Vita Health Co. Ltd., Winnipeg, MB), or coconut oil (Vita Health Co. Ltd., Winnipeg, MB), and mixing thoroughly. The diets were stored at 4°C for up to 3 days before addition to the fermenters.

3.2.2. Apparatus and culture conditions

The rumen simulation technique (RUSITEC) as described by Czerkawski and Breckenridge (1977) was used. The RUSITEC was equipped with eight fermenters, each of 820 mL nominal capacity. Inoculum was obtained from a ruminally fistulated Holstein steer fed a diet of 50% rolled wheat and 50% cubed forage (70% alfalfa hay and 30% barley straw). Ruminal fluid was filtered through four layers of cheese cloth upon collection. To begin the experiment, the fermenters were filled with 820 mL of filtered ruminal fluid, 20

g of solid digesta and 10 g of diet. Digesta and diet were contained in nylon bags (80 x 120 mm with pore size of 53 μm). The digesta bag was replaced by a feed bag (10 g) after 24 h. Thereafter, one bag was replaced daily, so that each feed bag remained in the fermenter for 48 h. Artificial saliva (McDougall 1948, modified by omitting $CaCl_2$ and $MgCl_2$, pH 8.2) was infused continuously into the fermenters at a dilution rate of 0.6 d^{-1}. The pH of the ruminal fluid in each fermenter was measured daily during feed bag exchange. Fermenters were fed for 8 d prior to commencement of data collection. The anaerobic technique of Hungate (1950), as modified by Bryant and Burkey (1953), was used consistently throughout the experiment. The steer from which inoculum for the RUSITEC was obtained was cared for in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.3. Diet, digestibility and digesta components

Fatty acid compositions of the three oils were determined by gas chromatographic analysis of their methyl esters (Metcalf et al. 1961) on a Varian 3700 with a 30 m x 0.25 mm ID SP-2330 fused-silica capillary column connected to a flame ionization detector. Helium was the carrier gas. Neutral detergent fibre (NDF) content in diets and digesta was determined as described by Goering and Van Soest (1970). In situ dry matter disappearance (ISDMD) at 48 h from feed bags was determined as described by Rode et al. (1986) on three days during the sampling period.

3.2.4. Fermentation gases and end-products

The gas in the collection bag connected to each fermenter was analysed for CH_4, H_2 and CO_2 on a Varian 3700 gas chromatograph equipped with a Porapak Q column (mesh size 80/100) coated with 6% FFAP and connected to a thermal conductivity detector. The carrier gas was N_2. Fifty μL of fermentation gas were injected and data were recorded and integrated on a Chromatopac C-R1B data system (Shimadzu Corporation, Kyoto, Japan). Amounts of CH_4, H_2, and CO_2 were determined against calibration curves. Total gas production was determined using a water displacement technique as described by Fedorak and Hrudey (1983).

Analyses for volatile fatty acids (VFA), ethanol, formate, lactate and succinate were conducted on 24-h accumulations of effluent from each fermenter. On sample collection day, sodium azide (as an aqueous stock solution) was placed in each effluent collection flask to yield approximately 0.1% (wt vol^{-1}) final concentration in a 24-h accumulation of effluent. Following thorough mixing, samples of accumulated effluent were withdrawn and transferred to storage at -40°C. Ethanol and VFA were analysed by gas chromatography on a Varian 3700 equipped with a DB-FFAP (15 m x 0.53 mm I.D; 1.0 micron film thickness) fused silica capillary column (J & W Scientific, Folsom, CA) connected to a flame ionization detector, with helium as the carrier gas. To determine lactate and succinate, samples were methylated with boron trifluoride-methanol in boiling water both for 5 min, extracted in chloroform and analysed on the same gas chromatography system. Formate was determined using the colorimetric method described by Sleat and Mah (1984).

3.2.5. Microbial counts

Upon removal of a feed bag (48 h) from the fermenter, excess fluid was gently squeezed out and the feed bag was inserted into a plastic bag containing 10 mL of artificial saliva (pH 7.0; McDougall 1948). The plastic bag was heat sealed, then pummelled (normal speed, 30 s) in a Stomacher 400 Laboratory blender (Seward Medical Limited, London, UK) to dislodge bacteria from feed particles. After pummelling, the liquid (designated feed bag liquid, FBL) was gently extracted from the feed bag into the plastic bag and collected for microbial counts and assay of enzymatic activity. Serial dilutions were prepared using an anaerobic salt solution (Bryant and Burkey 1953).

Total viable bacteria in FBL were enumerated by the roll tube count method (Hungate 1969). Cellulolytic, amylolytic and methanogenic bacterial populations were estimated by most probable number (MPN) technique with three tubes at each dilution (Koch 1981). The medium of Scott and Dehority (1965) was used for total bacterial enumeration. The same medium, modified to contain Whatman filter paper or soluble starch, was used to culture cellulolytic and amylolytic bacteria, respectively. Medium M2 (Hobson 1969), modified by adding 0.25% (wt vol^{-1}) sodium formate and 1% (vol vol^{-1}) of the trace vitamins solution of Scott and Dehority (1965), was used for determination of methanogenic bacterial populations. All cultures were incubated at 39°C. Bacterial colonies in roll tubes were counted after incubation for 5 d. Cultures for MPN determination of cellulolytic and methanogenic bacteria were assessed for digestion of the filter paper (visual inspection) and CH_4 production (headspace gas analysis), respectively, after 5 d of incubation. Cultures for

MPN determination of amylolytic bacteria were assessed for growth after 2 d.

3.2.6. Endoglucanase assay

Endoglucanase activity in FBL was determined using the soluble chromogenic substrate, ostazin brilliant red-hydroxyethyl cellulose (OBR-HEC) (Biely et al. 1985). Samples of FBL (0.1 mL) were mixed with 0.1 mL of 0.8% OBR-HEC (Sigma Chemical Co., St. Louis, MO) in 50 mM sodium phosphate buffer (pH 6.5) and incubated at 39°C for 2 h. Reactions were stopped by adding 0.8 mL of acetone-ethanol solution (1:2 vol vol^{-1}). Controls for each treatment were prepared by incubating OBR-HEC alone for 2 h, then adding FBL immediately prior to the acetone-ethanol solution. The mixtures were allowed to stand at room temperature for 30 min, and the precipitated substrates were removed by centrifugation (14,000 x g, 5 min). Absorbance at 550 nm was determined using a Stasar III spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH). Activity was standardized against a commercial endoglucanase from *Penicillium funiculosum* (EC 3.2.1.4., Sigma Chemical Co.) with 1 U of activity defined as the absorbance given by 1 U of commercial endoglucanase mL^{-1} h^{-1}.

3.2.7. Scanning electron microscopy

Samples of hay were collected from feed bags after 24 h of incubation in the RUSITEC. Specimens were fixed for 2 h in 5% (wt vol^{-1}) glutaraldehyde (J.B. EM Services

Inc., Dorval, QC) in 0.1 M cacodylate buffer (pH 7.2) containing 0.05% (wt vol^{-1}) ruthenium red. Specimens were then washed five times in 0.1 M cacodylate buffer with 0.05% (wt vol^{-1}) ruthenium red. The specimens were dehydrated in a graded ethanol series and critical-point dried, then mounted with silver paste on aluminium stubs and sputter coated with gold. They were viewed using a Hitachi S-570 scanning electron microscope at an accelerating voltage of 7-10kV and photographed on Ilford FP4 panchromatic film.

3.2.8. Statistical analysis

The experiment was conducted in a completely randomized 2 x 4 factorial arrangement with three replicates. Analysis of variance was performed using the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute Inc. 1990). Microbial counts were transformed using square root transformation in variance analysis (Steel and Torrie 1980). Main effects of diet (N = 2) and oil (N = 4), and their interaction (diet x oil, N = 8) were incorporated into the model. Effects of oil were compared separately among oil treatments, within and between diets. Comparisons among least-square means were performed by the procedure of possible difference (PDIFF, SAS Institute Inc. 1990).

3.3. Results

The canola and cod liver oils used in this study contained high proportions (89.7 and 75.2%, respectively) of unsaturated fatty acids (Table 3.1). Oleic acid ($C_{18:1}$) was the major component of canola oil, whereas cod liver oil was composed mainly of $C_{16:0}$, $C_{16:1}$, $C_{18:1}$, $C_{20:1}$, $C_{20:5}$, $C_{22:1}$ and $C_{22:6}$ fatty acids. In contrast, coconut oil contained very high concentrations of saturated fatty acids (88.7%), principally lauric (C_{12}) and myristic (C_{14}) acids.

The effects of lipid addition on diet digestion and production of gases are presented in Table 3.2. The extent to which addition of oil affected DM digestion depended on the type of diet. The 48-h ISDMD of the untreated concentrate diet was higher ($P < 0.05$) than that of the untreated grass hay diet (by 21%), but no difference ($P > 0.05$) in degradation of the NDF fraction was detected between the untreated concentrate and grass hay diets. Canola oil and cod liver oil did not affect ISDMD or NDF degradation in either diet. Coconut oil depressed ($P < 0.01$) ISDMD and NDF degradation of the grass hay diet (by 31% and 47%, respectively) but had no such effects on the concentrate diet.

Production of CH_4, CO_2 and H_2 (mmol g^{-1} DM digested) was higher ($P < 0.05$) from the untreated concentrate diet than from the untreated grass hay diet (Table 3.2). In general, lipids depressed ($P < 0.05$) CH_4 production. Coconut oil had the greatest effect, reducing CH_4 production from grass hay by 59%, and from concentrate by 85%. Canola oil and cod liver oil depressed CH_4 production by 26% and 29%, respectively, with the grass hay diet by 32% and 49% with the concentrate diet. Coconut oil reduced ($P < 0.05$) CO_2 production

from both diets, whereas canola oil and cod liver oil did not affect CO_2 production from either diet. Production of H_2 from both diets was increased three-fold ($P < 0.05$) by coconut oil. Canola oil decreased H_2 production ($P < 0.05$) from the concentrate diet only whereas cod liver oil did not affect ($P > 0.05$) H_2 production from either diet. Daily production of gases followed a similar pattern as production of gases per unit of DM digested.

The ratio of methane to carbon dioxide (CH_4:CO_2) did not differ between the untreated, canola oil- and cod liver oil-treated concentrate diet and hay diet. However, this ratio was lower ($P < 0.05$) when coconut oil was applied to concentrate than when it was added to hay (Table 3.2). In contrast, the CH_4:CO_2 ratio was not changed when lipids were added to the hay diet, but added lipids decreased ($P < 0.05$) this ratio from the concentrate diet.

The effects of lipid and diet as well as their interaction on the fermentative environment are presented in Table 3.3. In general, fluid pH was lower ($P < 0.01$) in fermenters receiving the concentrate diet than in those receiving hay, but was not affected by lipids. Production of VFA (mmol g^{-1} DMD) was greater ($P < 0.01$) from concentrate than from grass hay whereas lipids did not affect this parameter. There was an interaction ($P < 0.05$) between diet and lipid type for total VFA concentrations and the molar percentages of VFAs produced. A lower ($P < 0.05$) proportion of acetic acid, and a higher ($P < 0.05$) proportion of butyric acid were produced from the concentrate diet than from grass hay. Addition of lipids did not affect total VFA concentration, other than a coconut oil-mediated decrease in total VFA measured in effluent from fermenters receiving hay. Applied to grass hay, both oils increased the percentage of propionic acid, left butyric acid production

unchanged and decreased the percentage of acetic acid in total VFA. With the concentrate diet, propionic acid production was unaffected by these oils, butyric acid was decreased and the proportion of acetic acid was decreased by cod liver oil but not by canola oil. In contrast, coconut oil applied to grass hay decreased propionic acid, increased butyric acid and did not affect the proportion of acetic acid. On the concentrate diet, the shifts induced by coconut oil were reversed (propionate increased, butyrate increased) and the proportion of acetic acid was reduced as well. Levels of production of formate, lactate and succinate and ethanol on both diets were very low (Table 3.3).

The effects of the lipids on endoglucanase activity and bacterial populations in FBL are presented in Table 3.4. The effect of lipids on activity of endoglucanase varied ($P < 0.01$) with diet type. Endoglucanase activity was higher ($P < 0.01$) with untreated grass hay diet than with the untreated concentrate diet. Applied to the grass hay, canola and cod liver oil increased ($P < 0.05$) endoglucanase activity, whereas coconut oil depressed ($P < 0.05$) it. None of the lipids affected endoglucanase activity in fermenters receiving the concentrate diet. Canola and cod liver oils reduced ($P < 0.05$) the number of methanogenic bacteria, but did not affect other bacterial populations. Coconut oil lowered ($P < 0.01$) all four measured bacterial populations on both diets with the exception of cellulolytic bacteria in the concentrate diet.

Scanning electron microscopy (SEM) revealed that during 24 h of incubation in the RUSITEC, extensive disruption of the outermost layer of untreated (Fig. 3.1A) and canola oil-treated (Fig. 3.1B) orchardgrass hay, and substantial colonization of the internal surfaces. Disruption of the surface layers of cod liver oil-treated grass was less complete (Fig. 3.1C),

but colonization of internal tissues by a variety of morphotypes was nonetheless evident. By comparison, the outermost layer of orchardgrass hay treated with coconut oil was largely intact (Fig. 3.1D) and colonization of internal structures, observable in damaged areas, was sparse.

3.4. Discussion

3.4.1. Comparative assessment of RUSITEC system

Methane production from the untreated diets in this study (0.3 and 0.6 mmol g^{-1} DMD from untreated grass hay and concentrate, respectively) was much lower than that reported by Czerkawski and Breckenridge (1977) for an artificial rumen study involving diets of hay (1.7 mmol CH_4 g^{-1} DMD) and 80% concentrate:20% hay (1.4 mmol CH_4 g^{-1} DMD). However, CO_2 production (1.7 - 2.7 mmol g^{-1}) was within the range of that observed by Czerkawski and Breckenridge (1977, 2.9 - 5.5 mmol g^{-1}) when a 50% concentrate diet was fed to the RUSITEC. Moreover, the ratio of CH_4 to CO_2 (0.21 - 0.22) and total VFA production (4 - 5 mmol g^{-1} DM digested) were close to the values of CH_4:CO_2 (0.3 - 0.37) and total VFA production (5.7 - 7.0 mmol g^{-1} DM digested) reported by these authors. Wallace et al. (1981) reported productions of CH_4 at 1.4 mmol g^{-1} DM digested and total VFA at 7.7 mmol g^{-1} DM digested in an artificial rumen given 67% chopped hay and 33% bruised barley. Total VFA production in the present study were also within the ranges of values measured in vivo in which total VFA ranged from 6.5 to 8.8 mmol g^{-1} DM or OM digested in sheep (Weston and Hogan 1968; 1971; Siddons et al. 1984; Leng and Brett 1966), and from 3.8 to 11 mmol g^{-1} DM or OM digested in cattle (Davis 1967; Esdale et al. 1968; Rogers and Davis 1982; Siciliano-Jones and Murphy 1989). Moreover, the molar ratios of acetate:propionate:butyrate on the grass hay diet (63:26:11) and the concentrate diet (47:28:25) were well within the ranges of values found in the rumen (Hungate 1988).

Given these observations, comparisons among treatments in this study are expected to be valid.

3.4.2. Dietary digestibility

Coconut oil reduced NDF disappearance in the grass hay diet (Table 3.2), which is consistent with the findings of Sutton et al. (1983), who observed a 76% depression of NDF digestion in sheep fed a basal diet of 1/3 chopped hay and 2/3 concentrate mix pretreated with 6.7% coconut oil, compared to sheep fed untreated concentrate. Although coconut oil was applied at 10% in the present study, the observed depressions in NDF digestion were only 48% and 10%, on grass hay and concentrate diets, respectively. The variation between the two studies may be partly attributable to the greater dilution rate in the in vivo experiment, which would tend to reduce bacterial attachment to dietary fibre, and thus cause a greater depression in NDF digestion.

Dry matter disappearance from the concentrate diet was not depressed ($P > 0.05$) by coconut oil treatment. This is in agreement with other reports, in which long-chain fatty acids have had no inhibitory effect on the digestibility of starch or soluble carbohydrates (McAllan et al. 1983; Tamminga et al. 1983). This difference in the effectiveness of lipids in depressing feed digestion between fibre- and concentrate-based diets was reflective of the observed changes in microbial populations. Coconut oil reduced the cellulolytic bacterial population on the hay diet by about 700-fold, whereas it reduced the amylolytic bacterial population on the concentrate diet by only about 7-fold.

Khorasani et al. (1992) found no depression in the apparent digestibilities of DM and NDF of a concentrate:forage (60:40) diet when canola seed (40% fat) was added at levels up to 17.4% of the diet of dairy cows. Similarly, Sutton et al. (1975) found no depression in either OM or crude fibre digestion in sheep fed a basal diet of concentrates and hay supplemented with 3% cod liver oil. This agrees with the lack of effect of canola oil and cod liver oil supplementations on both DM and NDF digestion with both the grass hay diet and the concentrate diet in the present study. In our experiment, cod liver oil did not cause any greater degree of depression in fibre digestion than did canola oil, although cod liver oil had a higher degree of unsaturation due to fatty acids with multiple double bonds. Thus, the effect of lipids on fibre digestion is apparently dependent more on the properties of individual fatty acids than on the degree of unsaturation of fatty acids.

In the present study, the depression in DM and NDF digestion associated with coconut oil corresponded with a decrease in number of total bacteria and cellulolytic bacteria as well as in endoglucanase activity (Table 3.4). The increase in endoglucanase activity and its associated tendency for an increase in cellulolytic bacterial numbers induced by canola oil and cod liver oil was unexpected. However, canola and cod liver oil induced increases in endoglucanase activity and numbers of cellulolytic bacteria corresponded and may be responsible for the numerical increase in the DM and NDF digestibility of the grass hay diet. These observations are contradictory to the general concept that unsaturated fatty acids have the most adverse effect on fibrolytic bacteria (Henderson 1973; Maczulak et al. 1981). Henderson (1973) found that growth of the cellulolytic bacteria *Ruminococcus* spp. was more strongly inhibited by oleic acid than by lauric and myristic acids. Furthermore, Tesfa (1992)

reported that rapeseed oil decreased carboxylmethylcellulase and xylanase activities of rumen digesta, as well as the digestibilities of DM, NDF and ADF in the rumen of Friesian bulls. The mechanism by which canola oil and cod liver oil enhanced the endoglucanase activity is not known, but it could be associated with stimulating the growth of cellulolytic bacteria (Table 3.4).

The greater decrease in cellulolytic and total bacterial population mediated by coconut oil than by the other two oils in the RUSITEC may be associated with the oil causing detachment or preventing attachment of primary digestive bacteria onto feed particles. Scanning electron microscopy demonstrated that there was no bacterial colonization on the surface of the coconut oil-treated fibre (Fig. 3.1D). Anything that prevents microorganisms attaching onto feed particles would result in an increased rate at which microorganisms are washed out. A combination of detachment or prevention of attachment with a direct toxic effect of coconut oil, therefore, would result in a reduction in cellulolytic bacteria in the RUSITEC. However, Ørskov et al. (1978) found that coating feed particles with lipids was in itself of little importance since in situ DM digestion of the dried grass in the rumen of sheep was not depressed by spraying tallow at levels up to 15% of diet. Therefore, they attributed depressed cellulose digestion with lipids to a direct toxicity to cellulolytic bacteria. Because lack of colonization could also be due to the dramatically lowered bacterial numbers associated with toxicity of lauric and myristic acids (Galbraith et al. 1971; Henderson 1973), it is not possible to determine if toxicity or detachment is responsible for the reduced digestibility associated with coconut oil.

3.4.3. Methane production

The depression in CH_4 production induced by the supplementary lipids generally agrees with reports in which long-chain fatty acids have depressed CH_4 production (Czerkawski and Breckenridge 1969; Demeyer et al. 1969). Moreover, the present study revealed that the extent to which lipids depressed CH_4 production depended on not only the type of lipids, but also the diets to which the lipids were applied. The degree of depression in CH_4 production induced by lipids was greater with a concentrate-based diet than with a hay diet (Table 3.2). With the hay diet, the depressions in CH_4 production induced by canola oil and cod liver oil (26 and 29%, respectively) were similar to depressions (27, 26, and 34%) caused by infusing 60 g d^{-1} of oleic, linoleic, and linolenic acids, respectively, into the rumen of sheep fed dried hay (Czerkawski et al. 1966a). However, the 59% depression caused by coconut oil was much higher than the corresponding values above. On the other hand, with the concentrate diet, the 31, 47 and 84% depressions in CH_4 production associated with canola oil, cod liver oil and coconut oil, respectively, were higher than the 25% depression observed in sheep following treatment of a basal diet (30% sugar-beet pulp, 20% decorticated groundnut meal, 30% barley meal, 10% oatmeal and 5% ground oat husk) with linseed oil (Czerkawski et al. 1966c).

This study confirmed that lipid-mediated depression in CH_4 production was not directly associated with the degree of unsaturation of the fatty acids. Assuming the canola oil, cod liver oil and coconut oil used in this study to be 85% fatty acids, their mean unsaturations (mmol double bonds per g of oil) were 3.71, 5.05 and 0.30, respectively.

Consumption of 1 mmol of H_2 in the hydrogenation of each mmol of double bonds would theoretically decrease production of CH_4 by 0.25 mmol. Thus, the daily doses (0.91 g) of canola oil, cod liver oil and coconut oil would cause CH_4 production to be decreased by 0.84, 1.15 and 0.07 mmol d^{-1}, respectively. However, the depressions actually effected by these oils were 0.37, 0.38 and 1.33 mmol d^{-1} on grass hay, and 1.74, 2.25 and 3.43 mmol d^{-1} on concentrate (Table 3.2). Depression of CH_4 production induced by coconut oil greatly exceeded the stoichiometric calculation on both diets, whereas with canola oil and cod liver oil the reduction was lower than was expected if all double bonds were hydrogenated on grass hay, and approximately double the stoichiometric value on concentrate. Clearly more factors are involved than the degree of unsaturation.

In the present study, depressed CH_4 production with supplementation of canola oil, cod liver oil and coconut oil corresponded with a lowered methanogenic population (Fig. 3.2). The non-linear relationship between methane production and methanogen population may reflect the different degrees to which the added oils are toxic to methanogens. Addition of oils did not depress diet DM or NDF digestibility even though CH_4 production was reduced, with the exception of when coconut oil was added to the hay diet. This observation agrees with the observation of Czerkawski et al. (1966a,b,c) in which the extent of depression in CH_4 production with supplementation of fatty acids was far greater than that of depression in fibre digestibility in sheep. Lack of correlation between CH_4 production and feed digestibility confirms that methanogenesis is not the only route for electron deposition in continuous mixed culture systems.

3.5. Conclusions and Implications

Coconut oil supplementation reduced methane production from both diets, but to a greater degree for concentrate than hay. This reduction in methane production corresponded with a decrease in DM digestion for the hay diet, but not the concentrate diet. Supplementation of canola oil and cod liver oil also depressed methane production to a greater degree in the concentrate diet than in the hay diet, but these oils did not have a detrimental effect on the digestion of either diet. These results agree with the previous work that the C_{12} and C_{14} fatty acids in coconut oil are inhibitory to methanogens, cellulolytic and amylolytic bacteria. Biohydrogenation alone could not account for the reduction in methane production with canola oil and cod liver oil supplementation and at least a portion of this decrease appeared to be due to a shift in fermentation towards a greater production of propionate. Electron microscopy revealed that coating of the feed with oil and prevention of bacterial attachment was not responsible for reduced methane production in the hay diet supplemented with canola or cod liver oil. In the present study, it was concluded that a direct toxic effect of specific fatty acids on methanogens is the major factor responsible for the reduction in methane production with oil supplementation.

Table 3.1. Fatty acid composition (% by weight of total fatty acids) of the lipids

Fatty acid		Canola oil		Cod liver oil		Coconut oil	
		Mean	SD[z]	Mean	SD	Mean	SD
$C_{8:0}$	Caprylic	0	0	0	0	7.64	0.08
$C_{10:0}$	Capric	0	0	0	0	6.51	0.06
$C_{12:0}$	Lauric	0	0	0	0	46.64	0.74
$C_{14:0}$	Myristic	0	0	4.54	0.06	17.16	0.26
$C_{16:0}$	Palmitic	4.05	0.10	10.83	0.21	8.15	0.10
$C_{18:0}$	Stearic	2.25	0.02	2.17	0.02	2.55	0.05
$C_{20:0}$	Arachidic	0.77	0.06	0	0	0	0
$C_{16:1}$	Palmitoleic	0	0	9.25	0.02	0	0
$C_{18:1}$	Oleic	63.72	0.76	19.76	0.68	6.39	0.03
$C_{18:2}$	Linoleic	16.61	0.12	2.02	0.02	1.77	0.02
$C_{18:3}$	Linolenic	8.27	0.05	1.20	0.08	0	0
$C_{18:4}$		0	0	2.86	0.02	0	0
$C_{20:1}$	Gadoleic	1.14	0.02	12.40	0.23	0	0
$C_{20:2}$		0	0	0.29	0.01	0	0
$C_{20:4}$	Arachidonic	0	0	0.35	0.01	0	0
$C_{20:5}$		0	0	8.35	0.05	0	0
$C_{22:1}$	Erucic	0	0	6.71	0.07	0	0
$C_{22:5}$		0	0	1.09	0.01	0	0
$C_{22:6}$		0	0	10.87	0.15	0	0
Total saturated fatty acid:		7.07	0.17	17.55	0.29	88.66	1.19
Total unsaturated fatty acid:		89.74	0.90	75.17	0.41	8.16	0.05
Unidentified compounds:		3.19	0.02	7.28	0.12	3.18	0.09

[z]SD: standard deviation of the mean of triplicate measurements.

Table 3.2. Effect of dietary lipids on dry matter (DM) and neutral detergent fibre (NDF) disappearance and production of fermentation gases in the artificial rumen system (n = 3)

	Grass hay				Concentrate[z]					Significances		
Lipid[y]	CON	CAN	COD	COC	CON	CAN	COD	COC	SE	F[x]	L[x]	FxL
DM disappearance (%)	55.0b	59.1ab	61.1ab	38.1c	66.6a	55.4ab	55.4ab	57.1ab	3.7	NS	*	*
NDF disappearance (%)	49.5a	55.6a	54.9a	25.6b	56.4a	50.3a	50.5a	50.9a	3.7	NS	*	*
Gas production per digestible DM (mmol g^{-1})												
CH_4	0.34c	0.25d	0.24d	0.14e	0.58a	0.40b	0.31bcd	0.09e	0.05	**	**	**
CO_2	1.65b	1.44b	1.39b	0.68c	2.70a	3.20a	3.33a	1.75b	0.21	**	**	NS
H_2	0.002c	0.003c	0.003c	0.010b	0.010b	0.005c	0.007b	0.044a	0.001	**	**	**
Gas production (mmol d^{-1}):												
CH_4	1.84b	1.47b	1.46b	0.51c	3.94a	2.20b	1.69b	0.49c	0.43	**	**	*
CO_2	8.98b	8.55b	8.53c	2.59c	17.92a	17.71a	18.28a	9.97b	0.73	**	**	NS
H_2	0.01c	0.02bc	0.02bc	0.04b	0.06bc	0.03bc	0.04b	0.25a	0.006	**	**	**
CH_4:CO_2	0.21ab	0.17abc	0.17abc	0.20ab	0.22a	0.12bcd	0.09cd	0.05d	0.03	**	*	NS

[z] 90% wheat plus 10% grass hay.
[y] CON=control; CAN=canola oil; COD=cod liver oil; COC=coconut oil.
[x] F=feed; L=lipids.
NS = nonsignificant; * = P<0.05; ** = P<0.01.
a-e: Within a row, values not followed by the same letter differ ($P < 0.05$).

Table 3.3. Effect of dietary lipids on pH and profile of fermentation products in the fluid in the artificial rumen system (n = 3)

	Grass hay				Concentrate[z]					Significances		
Lipid[y]	CON	CAN	COD	COC	CON	CAN	COD	COC	SE	F[x]	L[x]	FxL
Effluent (mL d^{-1})	457.1ab	460.7ab	448.5b	452.9ab	487.1a	488.7a	488.6a	475.0ab	11.3	**	NS	NS
pH	7.15a	7.10a	7.10a	7.20a	6.65b	6.80b	6.85b	6.75b	0.75	**	NS	NS
Total VFA production per digestible DM (mmoles g^{-1})												
	4.0b	3.9b	3.9b	3.6b	5.1a	5.4a	5.8a	5.5a	0.3	**	NS	NS
Fermentation product concentration (mM)												
Total VFA	46.9c	50.2c	53.1bc	30.1d	69.0a	61.1ab	65.8a	65.7a	3.2	**	*	*
Formate	0.4b	0.4b	0.7b	0.4b	nd[w]	nd	nd	1.5a	0.3	NS	NS	NS
Lactate	nd	nd	nd	0.4	nd	nd	nd	0.4	0.001	NS	**	NS
Succinate	nd	nd	0.1b	1.3a	0.2b	0.2b	nd	1.4a	0.09	NS	**	**
Ethanol	1.8ab	2.0ab	1.9b	2.9ab	2.8ab	2.1ab	2.6ab	3.9a	0.62	NS	NS	NS
VFAs (molar %):												
Acetate	57.67a	53.46b	51.73b	55.98a	41.03c	39.36c	36.42d	36.10d	0.73	**	*	*
Propionate	24.10c	29.89b	30.04b	19.90d	24.18c	26.43c	28.56bc	32.92a	0.83	**	**	**
Butyrate	10.44d	8.89d	10.01d	15.28c	21.42a	18.79b	18.78b	16.15c	0.52	**	*	**

[z] 90% wheat plus 10% grass hay.
[y] CON=control; CAN=canola oil; COD=cod liver oil; COC=coconut oil.
[x] F = feed; L = lipids.
[w] nd = nondetectible.
NS = nonsignificant; * = P<0.05; ** = P<0.01.
a-d: Within a row, values not followed by the same letter differ ($P < 0.05$).

Table 3.4. Effect of dietary lipids on endoglucanase activity and ruminal microbial population in the feed particles in the artificial rumen system (n = 3)

	Grass hay				Concentrate[z]					Significances		
Lipid[y]	CON	CAN	COD	COC	CON	CAN	COD	COC	SE	F[x]	L[x]	FxL
Endoglucanase activity (unit mL^{-1} h^{-1})												
	1.05b	2.18a	2.42a	0.14c	0.29c	0.26c	0.18c	0.10c	0.12	**	**	**
Total bacteria (x 10^8 mL^{-1})[w]												
	124.8b	115.5b	150.1b	5.2d	200.5a	194.6ab	156.3ab	48.3c	0.6	**	**	NS
Cellulolytic bacteria (x 10^5 mL^{-1})[w]												
	76.0a	110.0a	112.8a	0.1b	5.9b	7.6b	5.9b	0.1b	1.8	**	**	*
Amylolytic bacteria (x 10^8 mL^{-1})[w]												
	125.0a	113.9a	107.6a	5.7c	139.2a	157.2a	110.6a	21.0b	1.0	NS	**	NS
Methanogens (x 10^4 mL^{-1})[w]												
	125.7b	15.7c	13.6c	0.1dc	474.8a	136.0b	96.4bc	0.1dc	4.0	**	**	NS

[z]90% wheat plus 10% grass hay.
[y]CON=control; CAN=canola oil; COD=cod liver oil; COC=coconut oil.
[x] F = feed; L = lipid.
[w]Means were actual bacterial counts, whereas the variance analysis was based on a square root transformation of actual bacterial counts.
NS = nonsignificant; * = P<0.05; ** = P<0.01.
a-d: Within a row, values not followed by the same letter differ ($P < 0.05$).

Fig. 3.1. Scanning electron micrographs of grass hay which was untreated (A), or treated with 10% (wt wt^{-1}) canola oil (B), cod liver oil (C) or coconut oil (D) and incubated in the RUSITEC for 24 h. The outermost layer of the grass is missing entirely in A and B, and only partially present in C. In contrast, the outer layer in coconut oil-treated grass hay was relatively undisturbed in most areas. Colonization of internal tissues by a variety of bacterial morphotypes was readily apparent on untreated, canola oil- and cod liver oil-treated samples (A, B, C), but was evident only at damage sites in coconut oil-treated hay, and was notably sparse (D). Bars = 5 μm.

A
B
C
D

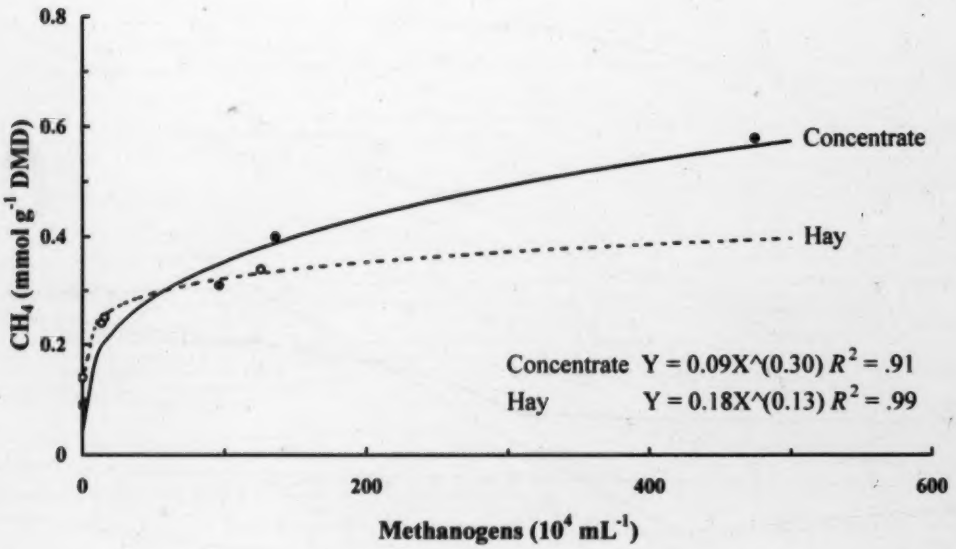

Fig. 3.2. The relationship between methane production and methanogenic population in a rumen simulation technique system with a orchardgrass hay-based diet or a concentrate-based diet (90% wheat + 10% hay).

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4. THE EFFECT OF EXOGENOUS FIBROLYTIC ENZYMES, α-BROMO-ETHANESULFONATE AND MONENSIN ON DIGESTIBILITY OF GRASS HAY AND METHANE PRODUCTION IN THE RUSITEC

4.1. Introduction

Ruminants fed forages exhibit reduced productivity, relative to those fed concentrate-based diets. This has been attributed to (i) lower digestibility of the feedstuffs and (ii) less efficient utilization of the energy in forages, arising from production of more acetate and methane from roughages than from concentrates during ruminal fermentation. Productivity of roughage-fed ruminants might be improved by implementing strategies to manipulate ruminal fermentation to increase fibre digestion and propionate production, and to depress methane production.

In recent years the newly exploited fibrolytic enzymes, which have the potential to substantially improve the utilization of fibrous feeds, prompted a renewed interest in feed enzymes for ruminant animals. Early studies showed that exogenous fibrolytic enzymes increased cellulose digestibility in vitro (Vandevoorde and Verstraete, 1987) and in vivo (Perry et al., 1960), but these enzyme preparations did not improve feed conversion efficiency in cattle (Leatherwood et al., 1960, Perry et al., 1960). More recent studies, however, demonstrated that pretreatment of hay with exogenous fibrolytic enzymes improved milk yield (Konno et al., 1993; Lewis et al., 1995; Stokes and Zheng, 1995) and live weight gain of cattle (Konno et al., 1993; Beauchemin et al., 1995). It has been found that the exogenous fungal fibrolytic enzymes effectively hydrolyse structural carbohydrates (McHan,

1986; Stokes, 1992) and yield more substrate for lactic acid-producing microorganisms during ensiling (Stokes, 1992). These findings imply that the improved productivities of ruminant animals fed forages treated with exogenous fibrolytic enzymes are partly attributed to an increase in availability of simple sugars which can be utilized by animals and/or ruminal lactate- and propionate-producing bacteria. Increasing competitiveness of ruminal lactate- and propionate-producing bacteria with acetate-producers can reduce ruminal methane production. However, addition of fungal cellulase to feeds was reported to increase methane production in vitro (Vandevoorde and Verstraete, 1987).

Research concerning the effect of supplementary fibrolytic enzymes on specific rumen microbial populations and on the ruminal fermentative process is limited. In mutualistic relationships with other microorganisms in the rumen and other anaerobic habitats, methanogens remove hydrogen, which renders the degradation of organic matter thermodynamically favourable in anaerobic fermentation. Thus, inhibition of methanogenesis would be expected to depress digestion of fibre in the rumen. In an earlier study in our laboratory, however, methane production in the artificial rumen system was significantly depressed by canola and cod liver oil without a decrease in dry matter (DM) digestion (Chapter 3). Similarly, Czerkawski et al. (1966a, 1966b) found that in sheep fed oleic and linoleic acids, cellulose digestion fell by 2%, whereas methane production was depressed by 39 and 43%, respectively. These observations confirm that methanogenesis is not the sole route for electron disposal in the rumen. Using methanogenic inhibitors to reduce methane production in the ruminal ecosystem therefore may not necessarily be at the expense of feed efficiency; in fact, an improvement in the utilization of dietary energy by

ruminants may occur when such products are used due to a reduction in the energy lost as methane.

α-Bromoethanesulfonate (BES), an analogue of coenzyme M (CoM) in methanogenic bacteria, has been studied as a specific inhibitor of methanogenesis (Taylor and Wolfe, 1974). A study with *Methanobacterium thermoautotrophicum* demonstrated that methanogenesis was inhibited by 50% by 7.9 μM BES, and was completely inhibited by 500 μM BES (Gunsalus et al., 1978). In addition, BES was found to be a potent inhibitor of the ruminal methanogen, *Methanobacterium ruminantium*, which requires an exogenous supply of CoM (Taylor et al., 1974). Uptake of HS-CoM by whole cells of *M. ruminantium* was completely inhibited by 1 μM BES (Balch and Wolfe, 1979). Martin and Macy (1985) demonstrated that 30 μM BES decreased methanogenesis by 76% in mixed cultures of ruminal fluid. The effect of BES on the relationship between digestibility and methane production has not been examined either in vitro or in vivo.

This experiment was conducted to examine the effect of supplementary fibrolytic enzymes and BES on fibre digestion and methane production. Monensin, a well known inhibitor of methanogenesis, was included as a positive control.

4.2. Materials and Methods

4.2.1. Experimental apparatus and operation

Two rumen simulation technique (RUSITEC) units (Czerkawski and Breckenridge, 1977), each equipped with eight fermenters (nominal volume 820 ml), were used in this study. Inoculum for the RUSITEC was obtained from a ruminally fistulated Holstein steer fed cubed alfalfa hay:barley straw (70:30 DM basis). This animal was cared for in accordance with the guidelines set by the Canadian Council on Animal Care (1993). Fermenters were initially filled with 820 ml of rumen fluid which had been filtered through four layers of cheesecloth, two nylon bags (203 × 102 mm; pore size 50 μm) each containing 10 g of solid rumen digesta, and a third bag containing 10 g of diet. After 24 h, the two inoculum bags were replaced with a second feed bag. Daily thereafter, the feed bag of longest residence was replaced, so that all feed was retained in the fermenter for 48 h. Artificial saliva (pH 8.2; McDougall, 1948) was continuously infused into the fermenters at a rate of 600 ml/d. At the time of feed bag exchange, fermentation fluid pH, total gas production and effluent from each fermenter were measured daily throughout the experiment. Eight days were allowed for equilibration of microbial populations prior to commencement of sample collection. The anaerobic technique of Hungate (1950), as modified by Bryant and Burkey (1953), was used consistently throughout the experiment.

4.2.2. Experimental diet and treatments

The experiment was designed as a complete randomized block with eight treatments: hay alone (**CON**) or pretreated with a fibrolytic enzyme mixture (**ENZ**), in combination with additions of no chemical, 25 μM monensin, 25 μM BES or 50 μM BES. The diet comprised early-bloom orchardgrass (*Dactylis glomerata*) hay ground to pass through a 4-mm screen. Enzyme-treated diet was prepared by spraying hay with the mixed enzyme solution (100 ml/kg DM) containing cellulase (200 g/l, Novo Nordisk, Denmark) and xylanase (200 g/l, Novo Nordisk, Denmark) at concentrations to yield final application rates of 30,000 nova cellulase units and 10,000 nova xylanase units, respectively, per kg hay DM. A control diet was prepared in the same manner with distilled water. Hays for both treatments were prepared 72 h prior to incubation in the RUSITEC and stored at 4°C.

Stock solutions of BES (Sigma Chemical Co., St. Louis, MO) were prepared in distilled water, whereas a stock solution of monensin (Hoechst Animal Health, Regina, SK) was prepared by dissolving the chemical in ethanol (>99.0%,v/v). Stock solutions were stored at 4°C. At the time of daily feed bag exchange, 1 ml of chemical stock solutions or distilled water was added into corresponding fermenter to yield initial concentrations of 25 μM monensin, and 25 or 50 μM BES in the cultures. Since the dilution rate of cultures was 0.7/d, the daily effective concentrations of chemicals at equilibrium varied from 48 μM at the time of dosing to 23 μM 24 h after dosing for the daily dosage of 25 μM monensin or BES, and from 97 to 47 μM for the daily dosage of 50 μM BES.

4.2.3. Sample collection and analysis

In situ dry matter disappearance (DMD) at 48 h from feed bags was determined from day 11 to day 13 of the experimental period. Feed bags withdrawn from each fermenter were washed under running warm (39°C) tap water until the water was clear. The feed bags were then dried at 105°C for 24 h. The feed bags for 0 h were soaked for 30 min in warm water (39°C), then washed and dried in the same manner as described above. The residues were pooled over 3 d and analysed for neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), using the methods of Van Soest et al. (1991). Hemicellulose content was calculated as the difference between NDF and ADF and cellulose as the difference between ADF and ADL.

Fermentation gas was collected using a gas collection bag which had been connected to fermenters. Total daily gas production from each fermenter was determined using a water displacement technique as described by Fedorak and Hrudey (1983) throughout the experiment. Concentrations of methane, carbon dioxide and hydrogen in the gas were measured for 7 days during day 9 to day 22 of the experimental period, using gas chromatography as described in Chatper 3.

Analysis of volatile fatty acids (VFAs) was conducted on 24-h accumulations of effluent containing sodium azide (0.1% final concentration, w/v) on day 20 to day 22 of the experiment. The effluent for each fermenter was collected once daily at the time of feed bag exchange. Effluent sample (4.0 ml) from each fermenter was taken in a screw capped vial containing 1.0 ml of 25% (w/w) phosphoric acid. Samples were immediately frozen and

stored at -40°C until analysis for VFAs by gas chromatography using the method as described by McAllister et al. (1990).

Microbial counts and enzyme assays were carried out on the liquid obtained from the 48-h incubated feed bag on d 14 and 16 of the experimental period. The feed bag liquid, prepared following the procedure described in Chapter 3, was diluted serially using an anaerobic solution of Bryant and Burkey (1953) for bacterial enumeration. Total viable bacteria in the liquid were enumerated by the roll tube count method (Hungate, 1969). Cellulolytic and methanogenic bacterial populations were estimated by most probable number (MPN) method (Koch, 1981). The medium of Scott and Dehority (1965) was used for total bacterial enumeration. The same medium, modified with addition of Whatman filter paper, was used to culture cellulolytic bacteria. Medium M2 (Hobson, 1969), modified by adding 0.25% (w/v) sodium formate and 1% (v/v) of trace vitamins solution of Scott and Dehority (1965), was used to culture methanogens. Bacterial populations were determined after 5-d incubation at 39°C. Numbers of total viable bacteria were directly determined from counts of bacterial colonies in roll tubes. Determination of cellulolytic bacterial population was assessed through visual inspection of filter paper degradation in cultures. The methanogenic population was estimated by measurement of methane production in the headspace of culture tubes using gas chromatography as described in Chapter 3.

β-Endoglucanase activity in the liquid from feed bags was determined with the soluble chromogenic substrate, ostazin brilliant red-hydroxyethylcellulose (Sigma Chemical Co., St. Louis, MO)(Biely et al., 1985), following the procedure described in Chapter 3. Xylanase activity in feed bag liquid was measured using beechwood 4-*O*-methyl-D-

glucurono-D-xylan (RBB-xylan) as a chromogenic substrate (Biely et al., 1985). Samples (0.1 ml) of the liquid from feed bags were mixed with 0.1 ml of 1.0% (w/v) RBB-xylan (Sigma Chemical Co., St. Louis, MO) in 50 mM sodium phosphate buffer (pH 6.5) and incubated for 30 min at 39°C. Reactions were stopped by adding 0.8 ml of the cooled (4°C) ethanol (>99.0%, v/v). Controls for each treatment were prepared by incubating RBB-xylan alone, then adding the liquid from feed bags immediately prior to addition of ethanol. The mixtures were allowed to stand at room temperature for 30 min, and the precipitated substrates were removed by centrifugation at 14,000 g for 5 min. Absorbance at 595 nm was determined using a Stasar III spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH). Activity was standardized against a commercial xylanase from *Aspergillus niger* (EC 3.2.1.8, Sigma Chemical Co., St. Louis, MO) with one unit of activity defined as the absorbance given by one unit of commercial xylanase per ml per h.

4.2.4. Statistical analysis

Analysis of variance was performed using the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute Inc., 1990). Main effect of enzyme (n = 2), chemical (n = 4) and RUSITEC (n = 2), and their interactions were incorporated into the initial analytical model. Microbial counts were transformed into a square root prior to variance analysis (Steel and Torrie, 1980). Differences among main effect means were tested with the method of the Student-Newman-Keuls (SNK). Comparisons among individual treatment means (least-square means) were performed by the procedure of possible

difference (PDIFF) (SAS Institute Inc., 1990).

4.3. Results and Discussion

4.3.1 The RUSITEC system

The mean methane production of 0.37 mmol per gram of digestible OM from untreated grass hay observed in the present study (Table 4.1) was similar to that recorded in an earlier RUSITEC study (Chapter 3), although the levels in both of these experiments were much lower than those reported in previous studies with the RUSITEC by Czerkawski and Breckenridge (1977) and Wallace et al. (1981). The comparative assessment of RUSITEC systems and the factors which could cause the lowered methane production with our RUSITEC systems have been discussed in Chapter 3.

4.3.2. Exogenous fibrolytic enzymes

Pretreatment of grass hay with fibrolytic enzymes increased ($P<0.01$) digestibilities of OM, cellulose and hemicellulose by 9, 15 and 20%, respectively (Table 4.1). These findings are consistent with the results of earlier in vitro studies (Hunt et al., 1995; Feng et al., 1996) and studies in cattle (Feng et al., 1996; Lewis et al., 1996). The increased digestibilities of cellulose and hemicellulose with enzyme treatment were consistent with the observed increases ($P<0.01$) in β-endoglucanase and xylanase activities (Table 4.2). Because application of the exogenous fibrolytic enzymes did not alter ($P>0.05$) either total bacterial or cellulolytic bacterial populations (Table 4.2), the increased digestibility of fibrous

components of hay with the enzyme treatment can probably be attributed to a direct catalytic action of the exogenous fibrolytic enzymes, rather than provision of essential branched fatty acids or other growth factors in the enzyme solutions to cellulolytic bacteria as has been previously suggested (Bowden and Church, 1959).

Pre-treating hay with fibrolytic enzymes increased ($P < 0.01$) methane production by approximately 43% as expressed in per unit of digestible OM (Table 4.1). This is consistent with an early report of Vandevoorde and Verstraete (1987), in which cellulase treatment induced an increase in methane production by approximately 23% from cellulose powder in vitro. The enzyme-induced increase in methane production observed in the present study corresponded to an overall increase ($P < 0.05$) in the methanogenic population (Table 4.2). However, the observation that enzyme treatment had no influence on total bacterial and fibrolytic bacterial populations raises a question concerning how enzyme treatment stimulated the growth of methanogens without stimulating the growth of fermentative bacteria since their activity is related to the food supply for methanogens. More research is needed to confirm the interactive relationship between methanogenic bacteria and fermentative microorganisms under the ruminal condition with feed digestion enhancer(s) such as fibrolytic enzymes.

Treatment of hay with fibrolytic enzymes did not alter either total VFA production per unit of digestible OM or the molar proportions of acetate and propionate, but it did increase ($P<0.01$) the molar proportion of butyrate (Table 4.3). These trends in VFA production patterns following enzyme treatment do not correspond with the increase in methane production. Decreased proportions of propionate (Miller and Wolin, 1973) would

be expected to accompany the increased methane production observed with enzyme pre-treatment of the grass hay.

4.3.3. α-Bromoethanesulfonate

No differences between BES levels of application were detected for the observed parameters other than for CO_2 and H_2 production. α-Bromoethanesulfonate did not influence the digestibilities of OM, cellulose and hemicellulose of the grass hay (Table 4.1). Similarly, it did not affect the population of cellulolytic bacteria and, therefore the activities of β-endoglucanase and xylanase (Table 4.2). To my knowledge, there has been no previous report concerning the effect of BES on forage digestion either in vitro or in vivo.

α-Bromoethanesulfonate depressed ($P<0.01$) methane production by 52% (Table 4.1). This observation is consistent with an earlier in vitro study (Martin and Macy, 1985). Immig et al. (1995) also found that BES significantly depressed methane production in sheep, but methane production was restored after 4 days of BES administration, suggesting methanogens in the rumen may have adapted to BES. α-Bromoethanesulfonate did not influence either the total bacterial population or cellulolytic bacterial population, but it significantly inhibited ($P<0.05$) the growth of methanogenic bacteria (Table 4.2). Therefore, a specific toxicity of BES to methanogenic bacteria was responsible for the reduction in methane production.

α-Bromoethanesulfonate did not change the total VFA production per unit of digestible OM or the molar proportions of acetate and propionate (Table 4.3). The decline

in methane production in fermenters receiving BES would seem to suggest that there should have been an increase in the molar proportion of propionate or in hydrogen production. This inconsistency suggests that alternative electron acceptors such as lactate, ethanol or succinate may have been produced during fermentation. Unfortunately, concentrations of these potential products were not measured.

4.3.4. Monensin

Monensin depressed ($P<0.01$) digestibilities of OM, cellulose and hemicellulose in untreated hay by 21, 30 and 21%, respectively, and, to a lesser extent, by 10, 24 and 13%, respectively, in enzyme-treated hay. Wallace et al. (1981) also observed declines in digestion of DM (19%), cellulose (48%) and hemicellulose (43%) when a RUSITEC was fed 10 mg of monensin daily. However, the effect of monensin on digestion has been inconsistent in the past. In various in vitro and in vivo studies, monensin has been reported to depress (Poos et al., 1979; Mir, 1989), to have a minimal effect (Dinius et al., 1976; Lemenager et al., 1978; Ricke et al., 1984; Faulkner et al., 1985) or to have no effect (Kone and Galyean, 1990; Duff et al., 1995), and even to increase (Wedegaertner and Johnson, 1983) fibre digestibility. This variability may arise from differences among these studies in dilution rates or the retention times of digesta in the rumen, which monensin may prolong (Lemenager et al., 1978). These factors remain relatively constant in the RUSITEC. The monensin-mediated depression in digestibilities of cellulose and hemicellulose was associated with a decrease ($P<0.05$) in cellulolytic bacterial population (Table 4.2). This observation is consistent with an earlier

pure culture study (Chen and Wolin, 1979) in which ruminal cellulolytic bacteria, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens* were sensitive to monensin, although the reduced population of cellulolytic bacteria may also have been related to a depressed methanogenesis (Miller and Wolin, 1973).

Monensin depressed ($P<0.05$) methane production per unit of digestible OM by 71% (Table 4.1). This is consistent with previous in vitro and in vivo studies (Van Nevel and Demeyer, 1977; Thornton and Owens, 1981; Wallace et al., 1981; Wedegaertner and Johnson, 1983; Martin and Macy, 1985). The observed depression in methane production corresponded to a decrease ($P<0.05$) in the population of methanogenic bacteria (Table 4.2). This observation agrees with an early pure culture study (Chen and Wolin, 1979) in which the growth of some ruminal methanogens such as *Methanobacterium bryantii*, *Methanobacterium formicicum* and *Methanosarcina barkeri* were inhibited by monensin.

Monensin did not change total VFA production, but it altered the molar proportions of VFAs, with an increase ($P<0.01$) in the proportion of propionate and a decrease ($P<0.05$) in the proportion of acetate (Table 4.3). These are consistent with other in vivo and in vitro observations (Durand 1982; Schelling 1984; Duff et al., 1995). The decreased molar proportion of acetate was associated with a decrease in the population of cellulolytic bacteria (Table 4.2). Ruminal cellulolytic bacteria *R. albus*, *R. flavefaciens* and *B. fibrisolvens* are sensitive to monensin (Chen and Wolin, 1979), and their major fermentative product is acetate (Stewart and Bryant, 1988). The increased molar proportion of propionate was related to an inhibition of methanogenic growth (Table 4.2) and methanogenesis (Table 4.1). Depressed methanogensis in the rumen results in the generation of propionate and other

reduced end product such as succinate (Chen and Wolin, 1977; Latham and Wolin, 1977).

4.3.5. Comparative potency of BES and monensin in methane depression

The present study indicated that monensin was a more potent inhibitor of methanogenesis than BES (71 *vs* 52% depression). This is opposite to the report of Martin and Macy (1985) in which monensin (10 ppm) and BES (30 μM) depressed methane production 16 and 76%, respectively. In my study, the concentration of monensin was about 70% higher, while the concentration of BES was about 17% lower than in their study. In addition, they evaporated methanol from monensin solution, which may have caused a decrease in solubilization of monensin in the culture media. Introduction of monensin in an ethanol solution form to fermenters in our study may have increased potency of monensin, but the possibility that ethanol directly influenced activity of methanogenic bacteria cannot be ruled out since its concentration could have reached 27 μM. Monensin decreased not only the methanogenic population, but also the fibrolytic bacterial population ($P<0.05$). Depressed growth of fibrolytic bacteria can aggravate the effect of monensin on methanogenesis. In contrast, BES only affected methanogenic bacteria and not other types of microorganisms. Therefore, it appears that BES may have potential as an anti-methanogenic agent.

4.4. Conclusions and Implications

Pretreatment of grass hay with fibrolytic enzymes increased fibre digestion and methane production in the RUSITEC. The mechanism(s) by which supplementary fibrolytic enzymes influenced ruminal fibre digestion and methanogenesis could not be determined, but this study suggests that the digestibility of roughage-based diets may be increased by enzyme supplementation. It was possible to decrease methane production with BES, without adversely affecting fibre digestibility, whereas fibre digestibility was decreased when monensin was used to depress methane production. The use of exogenous fungal fibrolytic enzymes and BES may prove to be effective agents for increasing the availability of metabolizable energy to ruminants consuming forages, although animal studies are required to assess the concerted effects of supplementary enzymes and BES as an inhibitor of methanogenesis in vivo.

Table 4.1. Effect of exogenous fibrolytic enzymes, α-bromoethanesulfonate (BES) and monensin (MON) on digestbilities of organic matter, cellulose and hemicellulose and on production of fermentation gases in RUSITEC fermenters receiving ground orchardgrass hay

	Chemical Treatment									Contrasts[x]				
					BES						Chemical			BES
	None		MON		25 μM		50 μM			ENZ *vs*	MON *vs*	BES *vs*	MON *vs*	25 *vs*
Diet[y]	CON	ENZ	CON	ENZ	CON	ENZ	CON	ENZ	SEM	CON	None	None	BES	50
Dilution rate (%/d)	72.9	73.6	73.0	74.6	72.1	74.0	74.4	73.8	3.1	NS	NS	NS	NS	NS
Digestibility (%):														
Organic matter	33.5*bc*	36.6*a*	26.6*d*	32.9*bc*	34.0*bc*	35.7*ab*	32.1*bc*	34.5*abc*	0.8	**	**	NS	**	NS
Cellulose	26.7*b*	30.8*a*	18.7*d*	23.5*c*	26.6*bc*	30.9*a*	25.4*c*	29.5*ab*	1.1	**	**	NS	**	NS
Hemicellulose	36.4*b*	43.5*a*	28.7*c*	37.9*b*	36.0*b*	42.2*a*	34.3*b*	41.2*a*	0.9	**	**	NS	**	NS
Gas production (mmol/d):														
CH_4	1.22*b*	1.95*a*	0.21*d*	0.62*bc*	0.66*bc*	0.80*bc*	0.58*c*	0.85*b*	0.08	**	**	**	**	NS
CO_2	3.38*bc*	6.84*a*	3.16*bc*	2.39*c*	2.23*c*	3.04*bc*	3.30*b*	4.11*b*	0.53	*	**	**	NS	NS
H_2	0.12*ab*	0.11*b*	0.14*a*	0.11*b*	0.11*b*	0.11*b*	0.13*ab*	0.14*a*	0.01	NS	NS	NS	NS	*
Gas production per digestible OM (mmol/g):														
CH_4	0.37*b*	0.53*a*	0.08*e*	0.19*d*	0.19*d*	0.23*cd*	0.18*d*	0.25*c*	0.02	**	*	**	**	NS
CO_2	1.01*bc*	1.87*a*	1.19*b*	0.73*bc*	0.66*c*	0.85*bc*	1.03*bc*	1.18*b*	0.15	NS	*	**	NS	*
H_2	0.036*ab*	0.030*b*	0.051*a*	0.034*b*	0.033*b*	0.032*b*	0.039*ab*	0.040*ab*	0.002	*	*	NS	*	*

[x]NS: not significant ($P > 0.05$); *: ($P < 0.05$); **: ($P < 0.01$).
[y]CON: Untreated orchardgrass hay; ENZ: Orchardgrass hay treated with cellulase and xylanase.
a-e: Within a row, values not followed by the same letter differ ($P < 0.05$).

Table 4. 2. Effect of exogenous fibrolytic enzymes, α-bromoethanesulfonate (BES) and monensin on (MON) feed particle-associated fibrolytic enzyme activities and bacterial populations in RUSITEC fermenters receiving ground orchardgrass hay

	Chemical Treatment									Contrasts[z]				
					BES						Chemical			BES
	None		MON		25 μM		50 μM			ENZ vs	MON vs	BES vs	MON vs	25 vs
Diet[y]	CON	ENZ	CON	ENZ	CON	ENZ	CON	ENZ	SEM	CON	None	None	BES	50
Endoglucanase (U/ml/h)[x]														
	0.2*b*	2.3*ab*	0.8*b*	3.9*a*	0.4*b*	2.0*ab*	0.3*b*	3.2*a*	0.7	**	NS	NS	NS	NS
Xylanase (U/ml/h)[x]														
	6.1*bc*	9.6*a*	4.8*c*	7.4*abc*	6.5*abc*	9.6*a*	6.1*bc*	9.1*ab*	1.0	**	NS	NS	*	NS
Total bacteria (× 10^{10}/ml)[w]														
	29.5	24.3	33.2	35.2	32.3	24.6	36.4	30.7	0.2	NS	NS	NS	NS	NS
Cellulolytic bacteria (× 10^{7}/ml)[w]														
	91.2*a*	61.7*abc*	39.5*bc*	36.9*c*	78.0*abc*	77.8*abc*	80.8*abc*	77.2*abc*	1.0	NS	*	NS	*	NS
Methanogens (× 10^{5}/ml)[w]														
	60.9*a*	72.9*a*	0.1*c*	19.7*abc*	6.5*bc*	47.0*ab*	12.2*abc*	47.1*ab*	2.3	*	**	*	*	NS

[z]NS: not significant ($P > 0.05$); *: ($P < 0.05$); **: ($P < 0.01$).
[y]CON: Untreated orchardgrass hay; ENZ: Orchardgrass hay treated with cellulase and xylanase.
[x]Units were defined as the absorbance given by one unit of commercial endoglucanase or xylanase/ml/h.
[w]Values shown are actual bacterial counts; analysis of variance was based on a square root transformation of the actual bacterial counts.
a-e: Within a row, values not followed by the same letter differ ($P < 0.05$).

Table 4.3. Effect of exogenous fibrolytic enzymes, α-bromoethanesulfonate (BES) and monensin on pH and volatile fatty acid profiles in RUSITEC fermenters receiving ground orchardgrass hay

	Chemical Treatment									Contrasts[x]				
					BES						Chemical			BES
	None		MON		25 μM		50 μM			ENZ vs	MON vs	BES vs	MON vs	25 vs
Diet[y]	CON	ENZ	CON	ENZ	CON	ENZ	CON	ENZ	SEM	CON	None	None	BES	50
pH	6.96	6.93	7.00	6.98	6.93	6.91	6.92	6.98	0.02	NS	NS	NS	NS	NS
Effluent production (ml/d)	598.0	603.5	598.5	612.0	591.5	607.0	610.0	605.0	25.6	NS	NS	NS	NS	NS
Total VFA concentration (mM)	21.2	22.1	16.7	18.7	23.1	21.9	22.3	18.2	3.5	NS	NS	NS	NS	NS
Total VFA production per digestible OM (mmol/g)	3.7	3.6	3.7	3.4	4.0	3.7	4.2	3.2	0.5	NS	NS	NS	NS	NS
VFA (molar %):														
Acetate	58.6*a*	58.1*a*	55.3*ab*	50.3*b*	58.0*a*	57.2*a*	58.1*a*	53.9*ab*	2.0	NS	*	NS	*	NS
Propionate	23.1*c*	20.5*c*	30.5*ab*	33.1*a*	23.9*bc*	20.6*c*	23.0*c*	22.0*c*	2.3	NS	**	NS	**	NS
Butyrate	18.2*bcd*	21.4*a*	14.2*d*	16.7*cd*	18.1*bcd*	22.2*ab*	18.9*bc*	24.0*a*	1.3	**	**	NS	**	NS

[x]NS: not significant ($P > 0.05$); *: ($P < 0.05$); **: ($P < 0.01$).
[y]CON: Untreated orchardgrass hay; ENZ: Orchardgrass hay treated with cellulase and xylanase.
a-e: Within a row, values not followed by the same letter differ ($P < 0.05$).

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5. INFLUENCE OF EXOGENOUS FIBROLYTIC ENZYMES AND α-BROMO-ETHANESULFONIC ACID ON SHEEP FED GRASS HAY: I. DIGESTION, METHANE PRODUCTION AND ENERGY METABOLISM

5.1. Introduction

Pre-treatment of forages with fungal fibrolytic enzymes has been shown to increase fibre digestibility (Dong , Chapter 4; Hunt et al., 1995; Feng et al., 1996; Lewis et al., 1996), food intake (Stokes and Zheng, 1995; Feng et al., 1996), milk yield (Konno et al., 1993; Lewis et al., 1995; Stokes and Zheng, 1995) and live weight gain of cattle (Konno et al., 1993; Beauchemin et al., 1995). On the other hand, in vitro studies have documented that application of exogenous fibrolytic enzymes also promotes methane production (Vandevoorde and Verstraete, 1987; Dong, Chapter 4). Increases in methane production could potentially offset any improvement in the utilisation of fibrolytic enzyme treated forages by ruminant animals. Simultaneous depression of methane production in the rumen would, therefore, maximise the beneficial effect that fibrolytic enzymes may have on improvement of forage utilisation.

A previous study has demonstrated that α-bromoethanesulfonic acid (BES), an analogue of coenzyme M (CoM) in methanogenic bacteria (Gunsalus et al., 1978), depressed methane production in the artificial rumen by as much as 50% with no detrimental effect on fibre degradation of grass hay (Dong, Chapter 4). Similarly, Martin and Macy (1985) reported that a 76% reduction of methane production was achieved in mixed cultures of rumen fluid containing 30 μM BES. A study in sheep also demonstrated that BES

effectively depressed methane production, but its effectiveness persisted for just 3 d (Immig et al., 1995). Although this implies that ruminal methanogens possess a very efficient mechanism(s) to protect their unique metabolic system, research into the inhibition of methane production in vivo with BES is very limited. The present experiment was therefore conducted to examine the effects of the exogenous fibrolytic enzymes and BES on fibre digestion, fermentation processes, methane production and energy metabolism in sheep.

5.2. Materials and Methods

5.2.1. Experimental animals and management

Six Suffolk ewes (85.5 ± 8.6 kg) consuming a grass hay-based diet were used to study the effect of exogenous fibrolytic enzymes and BES on digestion and methane production. Ewes were fitted with a ruminal cannula (5 cm I.D.) and a T-shaped flow-through cannula in the proximal duodenum. Surgery was performed in a sterile environment after feed had been withdrawn for 48 h. Procaine penicillin G (Pfizer, Point Claire-Duval, PQ) was injected intramuscularly twice daily (1×10^7 IU/injection) starting 24 h prior to surgery and continuing for 10 d after surgery. Anaesthesia was induced with intramuscular Atravet (Ayerst, Saint Laurent, PQ) and intravenous thiopentone sodium (Abbott, Montreal, PQ) and maintained by halothane and oxygen delivered through a semi-closed system with intermittent positive pressure ventilation. Ewes were allowed at least 15 days for recovery from surgery before the experiment was commenced. The experimental procedures with animals were approved by the Animal Care Committee of the Agriculture, Forestry and Home Economics Faculty. Animals were cared for in accordance with the guidelines set by the Canadian Council on Animal Care (1993).

Each experimental period was 43 d, comprising 14 d of adaptation followed by a 29-d sampling period. During the period of experiment, ewes were housed in individual metabolic crates and fed ad libitum four times daily (0800, 1400, 2000, 0200 hrs) with automatic feeders. Ewes were allowed free access to fresh water and trace-mineralized salt

(>99.0% NaCl, 0.015% I, 0.01% Co) (Sifto Canada Inc., Mississauga, ON) throughout the experiment. Body weight was measured at the beginning and the end of each experimental period. Between each experimental period, ewes were allowed 2 wk of exercise and recovery in floor pens. During the first 3 d of a recovery period, about 1 L of rumen fluid and 1 kg of rumen digesta from the ewes which were not given BES were transferred into the rumen of ewes which had previously been subjected to BES treatment.

5.2.2. Experimental diets and treatments

Late-bloom fescue (*Festuca* sp.) grass hay was used as a basal diet for ewes. Hay was chopped to pass through a 10-cm screen using a forage harvester (Sperry New Holland, New Holland, PA). The chopped hay was treated once daily at 2000 h by spraying either distilled water (**CON**) or an aqueous enzyme solution (**ENZ**) at a rate of 100 mL/kg DM. The enzyme solution contained 10% (vol/vol) enzyme "A" (FinnFeeds International Ltd., Marlborough, Wiltshire, U.K.) and 5% (vol/vol) enzyme "B" (FinnFeeds International Ltd., Marlborough, Wiltshire, U.K.) which provided β-endoglucanase activity of 36,450 units/kg hay DM and xylanase activity of 215,565 units/kg hay DM. Immediately after it was treated, 25% of the diet was fed to the ewes. The remaining portion was loaded on automatic feeders and fed to animals at 6, 12 and 18 h after treatment.

Ewes on BES treatment received 5 mL BES (Sigma Chemical Co., St. Louis, MO) solution (20 mg/mL) ruminally twice daily at the feeding times of 0800 and 2000 h. Ewes as controls for the BES treatment received 5 mL distilled water in the same manner.

5.2.3. Sample collections

Voluntary feed intake was determined during day 10 to day 23 of each experimental period. Daily amounts of feed fed and refused were weighed and recorded for each animal. Feed samples were collected once daily by hand sampling at multiple sites prior to feeding at 2000 h. Total refusals were collected once daily from each animal. Daily collections of feed and refusals were then composited on an equal proportion (20% of weight) over 14 days for each experimental period. All samples were placed in plastic bags and stored at 4°C until further chemical analysis.

Total collections of feces and urine were conducted during day 15 to 21 of each experimental period. Feces were collected into a container using a sloped metal screen to separate urine and feces. Urine was collected into a tub containing 20 mL of 6 M HCl, replenished daily, to prevent ammonia volatilization. Total outputs of feces and urine were measured and recorded once daily. Daily collections of feces and urine were pooled on equal proportion (20% of weight) over 7 days for each experimental period. Pooled samples were frozen and stored at –40°C until further analysis.

The activities of β-endoglucanase and xylanase associated with rumen liquid and digesta particles were assayed during day 22 to day 27 of each experimental period. About 500 g of rumen digesta were collected from the cranial, mid and caudal sections of the dorsal sac of the rumen. Rumen digesta were mixed thoroughly and two subsamples (about 100 g each) were taken for determination of DM and enzyme assays. The remaining rumen digesta was returned to the rumen. Samples used for enzyme assays were squeezed through four

layers of cheesecloth until no more liquid could be expressed. The liquid was collected into test tubes standing on ice and centrifuged at 29,000 x *g* at 4°C for 15 min to prepare supernatants for enzyme assays. About 2 g of the liquid-free rumen digesta prepared as described above were transferred into plastic containers standing on ice containing 20 mL of 10 mM NaH_2PO_4 buffer (pH 6.8) for measurement of enzyme activities associated with rumen particulate matters. Enzymes bound to rumen particulate matters were extracted by sonication following the procedure set by Silva et al. (1987). Digesta were disrupted by low sonication at 0°C for ten 30 s periods, with 30 s intervals between sonications to allow for cooling. Feed particles were removed by filtration and the filtrates were centrifuged at 29,000 x *g* at 4°C for 15 min. The supernatants were used for enzyme assay. Samples for determination of DM content were dried for 24 h at 105°C.

In situ dry matter disappearance (ISDMD) was conducted simultaneously during the days in which enzyme assays were conducted (day 22 to day 27). Triplicates of nylon bags (5 x 10 cm, 50 μm pore size), each containing 2.5 g DM of ground (≤1 mm) grass hay treated 24 h prior to ruminal incubation in the same manner as the hay fed directly to animals, were placed into the rumen either at 0800 h or at 2000 h and incubated for 12, 24, or 48 h. The nylon bags were withdrawn from the rumen and briefly rinsed with warm (39°C) tap water, transferred into a washing machine and washed with warm (39°C) water in the normal mode until the water was clear. Bags were then dried at 105°C for 24 h and ISDMD was calculated as DM weight loss that had occurred during incubation in the rumen.

Rumen fluid was collected from the ventral sac of the rumen with a strainer 2 h after each feeding on day 29, 30 and 31. Rumen fluid pH was immediately measured.

Subsamples (4.0 mL) were added to tubes containing 1.0 mL of 25% (wt/wt) phosphoric acid for volatile fatty acid (VFA) analysis. Samples were immediately frozen and stored at –40°C until analysis.

Cobalt ethylenediaminetetraacetic acid (**Co-EDTA**) and chromium mordanted fibre, which were prepared as described by Udén et al. (1980), were employed as liquid phase and solid phase markers, respectively, to measure rumen volumes and ruminal digesta kinetics. Five grams of Co-EDTA and 10 g of chromium modanted fibre were dosed intraruminally at 0800 h on day 32 with subsequent collection of rumen fluid and digesta over 72 h. Rumen fluid was collected through a strainer from the rumen ventral sac at 0, 1, 2, 3, 4, 6, 8, 10, 14, 18, 24, 30, 36, and 48 h post-dosing. About 100 g of rumen digesta were collected from the front, middle and rear of the dorsal sac of the rumen at 0, 2, 4, 6 10, 14,18, 24, 30, 36, 48, 56, and 72 h post-dosing using a long-handle tea spoon. Ruminal liquid dilution rate and particulate passage rate were determined by regressing the natural logarithm of ruminal marker concentrations after peak concentration over time. The absolute value of the slope was the estimated fractional rate of dilution or passage. Ruminal liquid volumes and particulate matter sizes were estimated according the following equation: pool size (L or g) = [amount of marker dosed (g)] / [marker concentration at time 0 extrapolated from the regression equation (g/L or g/g DM)].

Methane and heat production were determined during the last 9 days of each experimental period, using indirect calorimetry as described by Young et al. (1975). Two ewes, each housed in an individual metabolic crate in a respiration chamber, were measured at each time. Measurements were conducted continuously for two 36 h periods, each

consisting of 12 h equilibrium and 24 h of data collection. The Data Grabber system, developed by Godby and Gregory (personal communication) was used to collect data pertaining to respiratory rates, oxygen consumption, and carbon dioxide and methane production. Animal heat production was calculated according to the following formula: heat production (kcal/d) = 4.89 x O_2 consumed (L/d) (McLean, 1972).

5.2.4. Laboratory analyses

Unless specified otherwise, all samples for chemical analyses were dried at 60°C for 48 h and ground to pass through a 1-mm screen using a Wiley laboratory mill (model 4, Arthur H. Thomas Co., Philadelphia, PA). Dry matter content of all samples was determined by drying ground samples at 105°C for 24 h.

Nitrogen contents of feed and feces were determined with a nitrogen analyser (LECO model FP-428, St. Joseph, MI). Approximately 100 mg of feed or feces were weighed in a tin foil cup which was folded, compressed into a tablet form and completely combusted. Nitrogen content was determined from the nitrogen concentration in the combustion gases. Crude protein contents of feed and feces were then calculated by N content x 6.25. Gross energy (**GE**) contents of feed and feces were measured using a Parr adiabatic bomb calorimeter (Parr Instrument Co. Inc., Moline, IL) as described by AOAC (1990). Gross energy of urine was measured on freeze-dried urine in the same manner as described by AOAC (1990).

Neutral detergent fibre (**NDF**), acid detergent fibre (**ADF**) and acid detergent lignin

(**ADL**) were determined following the procedures described by Van Soest and Robertson (1980). Analysis of NDF was performed after preparing samples with a heat stable amylase (Sigma Chemical Co., St. Louis, MO) as suggested by Van Soest et al. (1991). Hemicellulose content was calculated as the difference between NDF and ADF and cellulose was the difference between ADF and ADL.

Volatile fatty acid concentrations in rumen fluid were measured using a Varian 3400 Gas Chromatograph (Varian, Sunnyvale, CA) equipped with a Stabilwax-DA column (30 m x .53 mm I. D. x .5 μm film thickness, Restex Co., Bellefonte, PA) and a flame ionization detector, with helium as the carrier gas. Samples were prepared following the procedure described by McAllister et al. (1990), modified with sample acidification using 25% (vol/vol) phosphoric acid.

Contents of cobalt (**Co**) in rumen fluid and chromium (**Cr**) in rumen digesta were determined by atomic absorption spectrophotometry (Pye Unican SP9, Scientific Instrument Co. of Philips, Cambridge, UK). Rumen fluid samples for Co analysis were thawed, and 15 mL of rumen fluid samples were centrifuged at 39,000 x *g* for 15 min. Concentrations of Co were determined at a wavelength of 240.7 nm after diluting the rumen fluid samples with 0.1 M HCl. Rumen digesta samples for Cr analysis were dried in an forced-air oven at 60°C for 96 h and ground using a coffee grinder. Samples (0.5 g) were pre-digested with 30 mL of 4 N HNO_3 at room temperature for 4 h and then incubated for 24 h at 75°C in water bath. Incubated samples were filtrated and supernatants were used to determine Cr concentrations at a wavelength of 357.9 nm.

β-Endoglucanase activity was assessed using ostazin brilliant red-

hydroxyethylcellulose (Sigma Chemical Co., St. Louis, MO) as a soluble chromogenic substrate (Biely et al., 1985), following the procedure described in Chapter 3. Xylanase activity was assayed with remazol brilliant blue-xylan (RBB-xylan) (Sigma Chemical Co., St. Louis, MO) as a soluble chromogenic substrate (Biely et al., 1985). Samples (0.1 mL) were mixed with 0.1 mL of 1.0% (wt/vol) RBB-xylan in 50 mM sodium phosphate buffer (pH 6.5) and incubated for 30 min at 39°C. Reactions were stopped by adding 0.8 mL of the cooled (4°C) ethanol (>99.0%, vol/vol). Controls for each treatment were prepared by incubating RBB-xylan alone, then adding the samples immediately prior to addition of ethanol. The mixtures were allowed to stand at room temperature for 30 min, and the precipitated substrates were removed by centrifugation at 14,000 x *g* for 5 min. Absorbance at 595 nm was determined using a spectrophotometer (Milton Roy, Spectronic 21, Rochester, N.Y.). Activity was standardized against a commercial xylanase from *Aspergillus niger* (EC 3.2.1.8, Sigma Chemical Co., St. Louis, MO) with one unit of activity defined as the absorbance given by one unit of commercial xylanase per mL per h.

5.2.5. Experimental design and statistical analysis

Six ewes were randomly arranged in a complete 4 x 4 and an incomplete 2 x 4 Latin square designs with 4 treatments in a 2 x 2 factorial arrangement. The treatments were hay alone or pretreated with fibrolytic enzymes combined without or with ruminal addition of BES. Variance analysis of data was performed with the general linear model procedure of the Statistical Analysis System (SAS Institute Inc., 1990). Variation sources included in the

initial analytic model were Latin squares (n = 2), experimental periods (n = 4), ewes (n = 6), enzymes (n = 2), BES (n = 2) and their interactions. Because of missing data for one ewe in the fourth period of the experiment, least square means for main effects were only computed with the GLM procedure. The procedure of repeated measurements was employed to analyse the data that were sampled over time. Differences between main experimental treatments were determined using the method of the Student-Newman-Keuls when variance component of main treatment effect was significant ($P < 0.05$) with the F-test. Comparisons among individual effect of treatments were performed with the possible difference procedure when interactions between treatments were significant ($P < 0.05$).

5.3. Results

No interactions ($P > 0.05$) between pre-treatment of hay with exogenous fibrolytic enzymes and ruminal addition of BES were detected for any of the parameters measured in the present study.

Pre-treatment of grass hay with exogenous fibrolytic enzymes did not influence ($P > 0.05$) the intakes and the total gastrointestinal tract digestibilities of DM, OM, NDF, ADF, cellulose, hemicellulose, CP and GE (Table 5.1 and Table 5.2). Similarly, pretreating hay with the enzymes did not affect ($P > 0.05$) in situ DM disappearances (ISDMD) measured after 12, 24 and 48 h ruminal incubation time although it tended to increase ($P = 0.08$) ISDMD measured at 0 h incubation (Table 5.3).

Pre-treatment of grass hay with exogenous fibrolytic enzymes increased the activities of β-endoglucanase ($P < 0.05$) and xylanase ($P < 0.01$) per mL per h in rumen liquid, but it did not enhance ($P > 0.05$) the activities of the fibrolytic enzymes per g DM per h in rumen digesta particles (Table 5.4). Application of fibrolytic enzymes to feed did not change the total ruminal activities of β-endoglucanase and xylanase in either rumen liquid or digesta particles (Table 5.4). It was clear that the digesta particle-associated fibrolytic enzyme activities were much higher ($P < 0.001$) than the liquid-associated activities.

Rumen pH was not changed ($P > 0.05$) when grass hay was treated with fibrolytic enzymes (Table 5.5). Similarly, neither concentrations nor molar proportions of VFAs were affected by fibrolytic enzymes (Table 5.5). Pretreatment of grass hay with fibrolytic enzymes had no effect ($P > 0.05$) on oxygen consumption, heat production or gross energy output in

urine (Table 5.6). Similarly, methane and carbon dioxide productions were not affected ($P > 0.05$) by fibrolytic enzymes.

Ruminal addition of BES did not affect ($P > 0.05$) the intakes of DM, OM, NDF, ADF, cellulose, hemicellulose, CP and GE (Table 5.1). Similarly, BES had no influence ($P > 0.05$) on the total gastrointestinal tract digestibilities of DM, OM, NDF, ADF, cellulose, hemicellulose, CP and GE (Table 5.2), or the in situ DM disappearance (Table 5.3). The addition of BES did not affect the fibrolytic enzyme activities per mL per h in rumen liquid or the activities per g DM per h in rumen digesta particles. Similarly, the total ruminal activities of β-endoglucanase and xylanase associated with either rumen liquid or digesta particles were not affected by enzyme treatment (Table 5.4).

α-Bromoethanesulfonic acid did not change ($P > 0.05$) rumen pH and VFA concentrations or molar proportion (Table 5.5). Similarly, ruminal addition of BES did not influence ($P > 0.05$) oxygen consumption, heat production and the gross energy output in urine (Table 5.6). Methane production was not depressed ($P > 0.05$) by ruminal addition of BES (Table 5.6).

5.4. Discussion

5.4.1. Exogenous fibrolytic enzymes

The voluntary intake of the ewes in present study (1.2% of body weight daily) was much lower than that expected (Table 5.1). As a consequence, live body weight of ewes dropped by 1.5 kg (±5.2 kg) during the experimental periods. Such a low intake may be attributed to the high cell wall content of the hay, which restricted intake of animals. In addition, the ewes were quite fat which would also reduce feed intake.

Pre-treating grass hay with exogenous fibrolytic enzymes did not enhance the voluntary intake of sheep (Table 5.1). This observation agrees with the results of earlier studies in sheep (McAllister et al., 1997) and in cattle (Lewis et al., 1995; Lewis et al., 1996). In contrast, other studies demonstrated that food intake was increased in cattle fed forages treated with fibrolytic enzymes (Perry et al., 1966; Chen et al., 1994; Stokes and Zheng, 1995; Feng et al., 1996; McAllister et al., 1997). Increased feed intake was correlated to increased digestion of feed (Perry et al., 1966; Chen et al., 1994; Feng et al., 1996) and/or passage of digesta particles (Feng et al., 1996). In the present study, the NDF content of the hay fed to the animals was high enough that an increase in intake would have been expected if digestion of the hay had been increased.

The total gastrointestinal tract digestibilities of grass hay were not improved by pretreatment of grass hay with the fibrolytic enzymes in present study (Table 5.2). Pretreatment of grass hay with fibrolytic enzymes did not improve in situ DM disappearances

of grass hay at 12, 24 and 48 h incubation, although it tended to increase (P = 0.08) DM disappearances at 0 h incubation (Table 5.3). These observations are consistent with the results from an earlier study with cattle (Hristov et al., 1998a, in press), in which pretreatment of a mixed diet consisting of rolled barley grain, corn silage and soybean meal with the same exogenous fibrolytic enzymes used in this experiment did not affect the rate or extent of in situ DM disappearance in the rumen and total tract digestibilities of the ration, although it increased the release of soluble reducing sugars and decreased NDF content of the diet prior to incubation. In the past, the effect of exogenous fibrolytic enzymes on forage digestion has been inconsistent. In various in vitro and in vivo studies, pre-treating forages or fibrous feedstuffs with exogenous fibrolytic enzymes has been reported to improve digestibility (Grainger and Stroud, 1960; Clark et al., 1961; Rovics and Ely, 1962; Van Walleghem et at., 1964; Perry et al., 1966; Galiev et al., 1982; Vandevoorde and Verstraete, 1987; Stokes, 1992; Fredeen and McQueen, 1993; Hunt et al., 1995; Feng et al., 1996; Lewis et al., 1996; Gwayumba and Christensen, 1997) or to have no effects, and even negative responses (Leatherwood et al., 1960; Perry et al., 1960; Theurer et al., 1963; Rust et al., 1965; Perry et al., 1966; Kennedy, 1987; Hristov et al., 1997; Krause et al., 1997; Wright et al., 1997). These inconsistencies may have arisen from differences in feed types (Beauchemin et al., 1995; Feng et al., 1996), method of enzyme application, and the enzyme stability and activity (Modyanov and Zel'ner, 1983).

The data from this study demonstrated that both β-endoglucanase and xylanase activities in rumen digesta particles were much higher than in rumen liquid, revealing the importance of binding of fibrolytic enzymes to feed particles. It has been evidenced that

binding of rumen microorganisms to their appropriate substrates is an absolute prerequisite for the digestion of plant cell walls (McAllister et al., 1994). However, application of the fibrolytic enzymes to feed did not change the activities of β-endoglucanase and xylanase associated with rumen digesta particles, although it did enhance the activities of the fibrolytic enzymes in rumen liquid (Table 5.4). Hristov et al. (1998b in press) found that the exogenous fibrolytic enzymes, which were of the same origin as used in the present study, were fairly resistant to rumen microbial degradation in vitro and in vivo. Therefore, the lack of response to the fibrolytic enzymes in the present study may be attributed partly, if not completely, to lack of binding to feed particles. Free enzymes in rumen fluid would have been washed out of the rumen.

Treatment of hay with the fibrolytic enzymes had no effect on methane production (Table 5.6). This observation contradicts the report of an early study using rumen simulation systems (Chapter 4), in which application of exogenous fibrolytic enzymes increased methane production by 43% along with a 10% increase in OM digestion of grass hay (Chapter 4). Application of the fibrolytic enzymes was shown to increase the population of ruminal methanogens along with fibre digestion in vitro, although it had no effect on the populations of either fibrolytic or total bacteria (Chapter 4). Although the mechanism(s) by which fibrolytic enzymes promoted the growth of methanogens was not determined in that study, it was postulated that crude preparations of the fungal enzymes provided an unknown growth factor(s) to methanogens rather than other microbes. Previous studies demonstrated that direct-fed crude preparations of *Aspergillus oryzae* increased the microbial populations in the rumen (Martin and Nisbet, 1992; Newbold, 1995; Kung, 1996). In the present study,

since fibrolytic enzymes had no effect on food intake, digestibility or digestion rate, any change in methane production with the fibrolytic enzymes might not have been expected.

5.4.2. α-Bromoethanesulfonic acid

Ruminal addition of BES did not affect the digestion of grass hay in sheep (Table 5.2 and Table 5.3), a result that is consistent with the findings of an earlier study in rumen simulation systems (Chapter 4). Consistently, addition of BES had no effect on the fibrolytic enzyme activities in the rumen (Table 5.4), implying BES in the rumen has no detrimental effect on fibrolytic bacteria. This finding is supported by a previous work with the RUSITEC in our laboratory, which addition of BES into fermenters did not change the population of fibrolytic bacteria and therefore the activities of fibrolytic enzymes (Chapter 4).

α-Bromoethanesulfonate did not effectively depress methane production in the present study (Table 5.6). This observation does not agree with findings of an earlier in vitro study by Martin and Macy (1985). Similarly, methane production was depressed by as much as 52% when BES was added continuously to the RUSITEC systems for 3 wk (Chapter 4). The inconsistency between previous in vitro studies and ours is not clear, but is probably related to adaptation of ruminal methanogens to BES after a prolonged administration in vivo. Indeed, mutants of *Methanosarcina* strain 227 have been discovered (Smith and Mah, 1981), which are resistant to 0.24 mM BES when previously exposed to a level of 0.024 mM. Smith and Mah (1981) discovered a strain of *Methanobacterium formicium* that is resistant

to 0.2 mM BES without any prior exposure. Immig et al. (1996) found that administration of BES dramatically decreased methane concentration of ruminal gases from about 40 to less 1% in sheep. However, methane concentration in ruminal gases reached 20% after BES was administrated for 4 days. These observations imply that there may exist species or strains of methanogens which are intrinsically resistant to BES in the rumen. These methanogens may or may not be cultivated within artificial rumen systems.

5.5. Implications

Pretreatment of grass hay with fibrolytic enzymes did not enhance fibre digestion in sheep. This lack of improvement in fibre digestion may be partly attributed to weak binding of the enzymes with feed particles. Ruminal application of BES did not effectively depress methane production in sheep. Failure to depress methane production may have resulted from adaptation of rumen methanogenic bacteria to BES.

Table 5.1. Effect of exogenous fibrolytic enzymes and α-bromoethanesulfonate (BES) on daily intakes of sheep fed long chopped grass hay

	Chemical[a]							
	None		BES			Probabilities[d]		
Item	CON[b]	ENZ[c]	CON	ENZ	SEM	BES	ENZ	BESxENZ
Body weight, kg	83.8	82.5	85.2	83.9	1.6	.40	.43	.99
Intake								
DM, kg/d	1.01	1.01	0.99	0.99	0.07	.87	.96	.96
OM, kg/d	0.95	0.94	0.93	0.93	0.07	.86	.90	.96
NDF, kg/d	0.71	0.71	0.70	0.70	0.05	.85	.98	.97
ADF, kg/d	0.35	0.35	0.35	0.35	0.03	.97	.96	.99
Cellulose, kg/d	0.27	0.27	0.27	0.26	0.02	.87	.86	.97
Hemicellulose, kg/d	0.36	0.36	0.35	0.35	0.03	.73	.99	.94
CP, kg/d	0.09	0.08	0.08	0.09	0.01	.99	.93	.91
GE, Mcal/d	4.844	4.805	4.753	4.732	0.347	.81	.93	.98

[a]None: No α-bromoethanesulfonate treatment; BES: α-Bromoethanesulfonate treatment.
[b]CON: Untreated grass hay.
[c]ENZ: Grass hay treated with cellulase and xylanase.
[d]Probability that treatment has no effect; BES: main effect of α-bromoethanesulfonate treatment; ENZ: main effect of enzyme treatment; BESxENZ: interaction between α-bromoethanesulfonate and enzyme treatmeants.

Table 5.2. Effect of exogenous fibrolytic enzymes and α-bromoethanesulfonate (BES) on apparent digestibilities (%) of long chopped grass hay in the total gastrointestinal tract of sheep fed long chopped grass hay

	Chemical[a]							
	None		BES			Probabilities[d]		
Item	CON[b]	ENZ[c]	CON	ENZ	SEM	BES	ENZ	BESxENZ
DM	49.7	48.6	47.4	47.9	1.8	.41	.86	.69
OM	53.4	52.4	51.1	51.8	1.7	.37	.93	.61
NDF	50.6	49.0	48.3	49.1	1.9	.53	.83	.55
ADF	40.8	38.3	39.6	40.4	2.0	.82	.66	.44
Cellulose	52.2	47.4	50.5	49.8	2.8	.90	.32	.51
Hemicellulose	60.0	59.3	57.0	57.7	2.3	.32	.99	.79
CP	50.6	50.0	48.1	48.5	2.2	.21	.94	.58
GE	50.3	49.3	46.8	48.1	1.9	.35	.95	.84

[a]None: No α-bromoethanesulfonate treatment; BES: α-Bromoethanesulfonate treatment.
[b]CON: Untreated grass hay.
[c]ENZ: Grass hay treated with cellulase and xylanase.
[d]Probability that treatment has no effect; BES: main effect of α-bromoethanesulfonate treatment; ENZ: main effect of enzyme treatment; BESxENZ: interaction between α-bromoethanesulfonate and enzyme treatmeants.

Table 5.3. Effect of exogenous fibrolytic enzymes and α-bromoethanesulfonate (BES) on in situ dry matter disappearance (%) in sheep fed long chopped grass hay

	Chemical[a]							
	None		BES			Probabilities[d]		
Time	CON[b]	ENZ[c]	CON	ENZ	SEM	BES	ENZ	BESxENZ
0	21.8	23.1	22.3	23.2	0.5	.65	.08	.76
12	46.2	46.3	47.0	46.5	1.5	.72	.90	.85
24	57.1	56.2	58.2	57.9	1.4	.32	.67	.84
48	68.7	68.2	69.4	68.6	0.8	.52	.44	.86

[a]None: No α-bromoethanesulfonate treatment; BES: α-Bromoethanesulfonate treatment.
[b]CON: Untreated grass hay.
[c]ENZ: Grass hay treated with cellulase and xylanase.
[d]Probability that treatment has no effect; BES: main effect of α-bromoethanesulfonate treatment; ENZ: main effect of enzyme treatment; BESxENZ: interaction between α-bromoethanesulfonate and enzyme treatmeants.

Table 5.4. Effect of exogenous fibrolytic enzymes and α-bromoethanesulfonate (BES) on the ruminal liquid volume, dry matter content and activities of β-endoglucanase and xylanase in sheep fed long chopped grass hay

	Chemical[a]							
	None		BES			Probabilities[d]		
Item	CON[b]	ENZ[c]	CON	ENZ	SEM	BES	ENZ	BESxENZ
Ruminal liquid volume, L	5.26	5.63	5.98	5.69	0.48	.43	.94	.53
Ruminal DM content, kg	1.07	1.16	0.88	1.24	0.21	.31	.79	.56
Activity in liquid phase, units/mL per h								
β-endoglucanase	0.027	0.028	0.024	0.030	0.001	.91	.05	.26
xylanase	0.305	0.347	0.324	0.341	0.010	.53	.01	.28
Activity in particulate phase, units/g DM per h								
β-endoglucanase	3.26	3.12	3.02	3.12	0.06	.08	.71	.11
xylanase	30.51	28.75	26.23	27.79	1.32	.09	.79	.20
Total activity in liquid phase, units (x 10^3)								
β-endoglucanase	0.14	0.16	0.14	0.17	0.02	.61	.22	.80
xylanase	1.58	1.92	1.93	1.97	0.18	.29	.29	.46
Total activity in particulate phase, units (x 10^3)								
β-endoglucanase	3.80	2.73	3.77	4.17	0.86	.43	.69	.44
xylanase	31.74	23.93	29.27	33.15	5.36	.54	.71	.33

[a]None: No α-bromoethanesulfonate treatment; BES: α-Bromoethanesulfonate treatment.
[b]CON: Untreated grass hay.
[c]ENZ: Grass hay treated with cellulase and xylanase.
[d]Probability that treatment has no effect; BES: main effect of α-bromoethanesulfonate treatment; ENZ: main effect of enzyme treatment; BESxENZ: interaction between α-bromoethanesulfonate and enzyme treatmeants.

Table 5.5. Effect of exogenous fibrolytic enzymes and α-bromoethanesulfonate (BES) on the ruminal pH and volatile fatty acid production in sheep fed long chopped grass hay

	Chemical[a]							
	None		BES			Probabilities[d]		
Item	CON[b]	ENZ[c]	CON	ENZ	SEM	BES	ENZ	BESxENZ
pH	6.86	6.83	6.83	6.81	0.02	.66	.63	.82
Volatile fatty acid concentration, mM								
Total	89.3	81.1	81.3	84.2	4.3	.56	.53	.25
Acetate	63.5	58.0	58.0	58.7	3.1	.44	.44	.37
Propionate	14.0	12.1	12.6	13.6	0.8	.90	.58	.10
Butyrate	9.3	8.5	8.2	9.3	0.4	.71	.72	.06
Valerate	0.69	0.64	0.63	0.71	0.03	.82	.69	.11
Caproinate	0.28	0.21	0.23	0.22	0.03	.57	.20	.27
Isobutyrate	0.71	0.72	0.73	0.74	0.03	.50	.80	.88
Isovalerate	0.86	0.94	0.88	0.85	0.05	.47	.66	.35
Volatile fatty acids, molar %								
Acetate	70.9	71.5	71.5	69.8	0.3	.09	.13	.01
Propionate	15.7	14.9	15.5	16.2	0.2	.04	.87	.01
Butyrate	10.5	10.5	10.0	11.0	0.3	.98	.09	.10

[a]None: No α-bromoethanesulfonate treatment; BES: α-Bromoethanesulfonate treatment.
[b]CON: Untreated grass hay.
[c]ENZ: Grass hay treated with cellulase and xylanase.
[d]Probability that treatment has no effect; BES: main effect of α-bromoethanesulfonate treatment; ENZ: main effect of enzyme treatment; BESxENZ: interaction between α-bromoethanesulfonate and enzyme treatmeants.

Table 5.6. Effect of exogenous fibrolytic enzymes and α-bromoethanesulfonate (BES) on methane and heat production in sheep fed long chopped grass hay

	Chemical[a]							
	None		BES			Probabilities[d]		
Item	CON[b]	ENZ[c]	CON	ENZ	SEM	BES	ENZ	BESxENZ
Oxygen consumption								
L/d	486.5	447.4	436.0	450.4	23.5	.31	.58	.35
L/kg OMI	520.0	506.8	477.6	504.1	48.1	.63	.88	.73
L/kg $BW^{0.75}$	17.6	16.4	15.6	16.4	1.0	.35	.82	.41
Carbon dioxide production								
L/d	527.3	535.3	537.8	513.9	38.0	.51	.24	.83
L/kg OMI	556.0	535.3	537.8	531.9	58.2	.73	.68	.98
L/kg $BW^{0.75}$	19.0	17.3	17.7	16.9	1.5	.55	.39	.82
Methane production								
L/d	32.2	29.7	27.9	26.4	2.6	.15	.41	.87
L/kg OMI	34.4	34.0	30.6	29.9	3.6	.27	.87	.97
L/kg $BW^{0.75}$	1.2	1.1	1.0	1.0	0.1	.15	.48	.74
Energy loss in methane								
Kcal/d	303.3	280.0	262.6	248.6	24.0	.15	.41	.87
Kcal/kg OMI	323.5	319.9	287.9	281.5	33.4	.27	.87	.97
Kcal/kg $BW^{0.75}$	11.1	10.2	9.4	9.2	0.9	.15	.47	.74
Energy loss in urine								
Kcal/d	174.4	120.6	125.9	134.6	16.8	.81	.56	.39
Kcal/kg OMI	160.8	156.8	141.5	147.2	16.9	.39	.96	.81
Kcal/kg $BW^{0.75}$	5.3	4.4	4.5	4.9	0.7	.77	.67	.43
Heat production								
Kcal/d	2385	2182	2108	2201	111	.25	.60	.28
Kcal/kg OMI	2549	2473	2316	2464	238	.60	.87	.70
Kcal/kg $BW^{0.75}$	86	80	76	80	5	.29	.85	.34

[a]None: No α-bromoethanesulfonate treatment; BES: α-Bromoethanesulfonate treatment.
[b]CON: Untreated grass hay.
[c]ENZ: Grass hay treated with cellulase and xylanase.
[d]Probability tha treatment has no effect; BES: main effect of α-bromoethanesulfonate treatment; ENZ: main effect of enzyme treatment; BESxENZ: interaction between α-bromoethanesulfonate and enzyme treatmeants.

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6. INFLUENCE OF EXOGENOUS FIBROLYTIC ENZYMES AND α-BROMO-ETHANESULFONATE ON SHEEP FED GRASS HAY: II. RUMEN DIGESTA KINETICS, NUTRIENT DIGESTION SITES, BACTERIAL OUTFLOW TO THE DUODENUM AND THEIR RELATIONS TO METHANE PRODUCTION

6.1. Introduction

Pretreatment of forages with exogenous fibrolytic enzymes have been shown to improve total tract fiber digestion and in situ fiber disappearance in cattle (Hunt et al., 1995; Feng et al., 1996; Lewis et al., 1996). On the other hand, earlier in vitro studies have demonstrated that supplementation of fibrolytic enzymes increased methane production along with increased fiber digestion (Vandevoorde and Verstraete, 1987; Chapter 4). Increases in methane production would offset the efficiency of fibrolytic enzymes in improvement of forage utilization by ruminants. However, the effect of exogenous fibrolytic enzymes on fiber digestion and methane production has yet been examined simultaneously in vivo.

Early in vitro studies have demonstrated that α-bromoethanesulfonic acid (BES), a specific competitive inhibitor of methanogens (Gunsalus et al., 1978), depressed methane production by 50-76% (Martin and Macy, 1985; Chapter 4). Similarly, a study with sheep also indicated that BES effectively depressed methane production, although methane production rose to normal level after three days of administration of BES (Immig et al., 1996). Reduction in methane production with BES would improve efficiency of feed energy utilization by ruminants and reduce contribution to greenhouse gases. On the other hand,

reduction in methane production could also reduce microbial protein synthesis in the rumen, since studies with axenic cultures have demonstrated that ruminal microbial growth is reduced when methanogens are absent (Miller and Wolin, 1973; Latham and Wolin, 1977; Marvin-Sikkema et al., 1990). In vivo studies regarding inhibition of ruminal methanogenesis with BES and its effect on microbial protein synthesis are limited.

This report, as a part of large animal experiment, focused on the study in the effect of exogenous fibrolytic enzymes and BES on rumen kinetics, outflows of nutrients and diaminopimelic acid (DAPA), and digestion sites. Relationships between methanogenesis, rumen microbial synthesis, and fibre digestion in and passage out of the rumen were also examined. Information concerning total tract digestion of the grass hay with fibrolytic enzymes and BES has been reported in the previous chapter.

6.2. Materials and Methods

The effects of exogenous fibrolytic enzymes and BES on digesta kinetics, site of digestion, methane production and DAPA flow to the duodenum were studied with six Suffolk ewes (85.5 ± 8.6 kg), each fitted with a ruminal cannula (5 cm I.D.) and a T-shaped flow-through cannula in the proximal duodenum. Ewes were randomly arranged into a complete 4 x 4 Latin square and an incomplete 2 x 4 Latin square design with four treatments in a 2 x 2 factorial arrangement. The treatments were hay alone (**CON**) or pre-treated with exogenous fibrolytic enzymes (**ENZ**) combined without or with ruminal administration of BES. Ewes were cannulated following the procedures described previously (Chapter 5). Animals were cared for following the guidelines set by the Canadian Council on Animal Care (1993) throughout the experiment.

The experiment consisted of four periods, each of 43 d duration. Ewes were allowed to adapt to their diet for 14 d prior to commencement of sample collection. Ewes were housed in individual metabolic crates and fed ad libitum four times daily at 0800, 1400, 2000, 0200 h with automatic feeders. Throughout the experiment, ewes were allowed free access to fresh water and trace-mineral salt. Between each experimental period, ewes were allowed 2 wk of exercise in floor pens. During the first 3 d of this period approximately 1 L of rumen fluid and 1 kg of rumen digesta from the ewes which were not on BES treatment were transferred into the rumen of ewes which had previously been subjected to BES treatment to minimize any residue effects of BES.

The diet for ewes consisted of late-bloom fescue (*Festuca* sp.) grass hay, which was

chopped to pass through a 10-cm screen before feeding. Enzyme treatment was prepared once daily at 2000 h by spraying an aqueous enzyme solution at a rate of 100 mL/kg DM containing 10 mL enzyme "A" and 5 mL enzyme "B" (FinnFeeds International Ltd., Marlborough, Wiltshire, U.K.) and yielding β-endoglucanase activity of 36,450 units/kg DM and xylanase activity of 215,565 units/kg DM. An equal amount of distilled water to that of the enzyme solution was sprayed on grass hay in the control treatment. Approximately 25% of the treated hay was offered to ewes immediately after preparation. The remaining material was loaded on automatic feeders and fed at 6 h intervals. Ewes on BES treatment received 5 mL BES (Sigma Chemical Co., St. Louis, MO) solution (20 mg/mL) intraruminally twice a day at 12 h intervals. Control animals received 5 mL distilled water in a same fashion as the BES treatment.

To estimate digesta DM flow to the duodenum, ytterbium (**Yb**) was employed as an external marker. A solution of $YbCl_3 \cdot H_2O$, containing 1 mg Yb/mL, was continuously infused into the rumen via a multichannel peristaltic pump from d 22 to d 29 at a rate of 144 mL/d. Collections of duodenal digesta and feces were conducted on d 27, 28 and 29 with collection intervals of 6 h. During collection of duodenal digesta, cannulas were opened for a maximum time period of 1 h and a maximum of 200 g of sample was obtained. Feces were collected into containers using a sloped metal screen to separate urine from feces and collected every 6 h. Samples of duodenal digesta and feces were pooled within sample type through collection times and days for each period on an equal proportion basis (20% of weight). Duodenal digesta was freeze dried, ground using a coffee grinder equipped with a cooling system utilizing running cool tap water (type A10, Janke & Kunkel KG, IKA

WERK, Staufen i. Breisgau, Germany), and stored at 4°C until analysis. Feces were oven dried at 60°C for 48 h, ground to pass through a 1-mm screen using a Wiley laboratory mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA), and stored at room temperature.

Ytterbium concentrations in duodenal digesta and feces were determined by atomic absorption spectrometry (Pye Unican SP2900, Scientific Instrument Co. of Philips, Cambridge, UK) using an acetylene and nitrous oxide flame as described by Siddons et al. (1985) after extraction of Yb from the ashed material with 20 mL of solution containing 2.25 M HCl, 2.25 M HNO_3 and 25.6 mM K^+ as KCl. Standards were made by addition of Yb atomic absorption standard solution (Aldrich Chemical Co. Inc., Milwaukee, WI) into the liquid which was obtained from fecal samples collected prior to dosing and subjected to processing in the same manner as other samples.

Digesta dry matter (DM) flow to the duodenum and fecal DM output were calculated as follows: DM output (g/d) = daily dose of Yb (g/d)/concentration of Yb in duodenal digesta or feces (g/g DM). The mean recovery of Yb in the feces was 91.6 ± 2.7% (n = 23).

Diaminopimelic acid was used as a bacterial marker. Concentrations of DAPA were determined using a HPLC (Varian 5000 Liquid Chromatogrph, Varian Instruments, CA, USA) following the procedure described by Dugan et al. (1992).

Digesta kinetics in the rumen was studied using cobalt ethylenediaminetetraacetic acid (**Co-EDTA**) and chromium mordanted fibre as liquid and solid phase markers, respectively. The markers were prepared following the procedure of Udén et al. (1980). Five gram of Co-EDTA and 10 g of chromium mordanted fibre were dosed intraruminally on day 32 with subsequent collection of rumen fluid and solid contents over 72 h. Rumen fluid was

collected through a strainer from the rumen ventral sac at 0, 1, 2, 3, 4, 6, 8, 10, 14, 18, 24, 30, 36, and 48 h post-dosing. Approximately 200 g of rumen contents were collected from the cranial, mid, and caudal of the dorsal sac of the rumen at 0, 2, 4, 6 10, 14, 18, 24, 30, 36, 48, 56, and 72 h post-dosing using a long-handled spoon. The samples were then mixed thoroughly and a sample of approximately 50 g was taken for analysis. The remaining portion of the sample was returned to the rumen. Ruminal liquid dilution rates and particulate matter passage rates were determined by regressing the natural logarithm of marker concentrations over time after peak concentration. The absolute value of the slope was taken as fractional rate of dilution or passage. Rumen pool sizes of fluid or particulate matter were estimated as follows: rumen pool size = dose of marker/marker concentration at time 0 as extrapolated from the regression equation.

Methane production was determined from d 35 to 43 of each experimental period, using indirect calorimetry techniques as described by Young et al. (1975). Two ewes, each housed in an individual metabolic crate in a respiration chamber, were measured at each time. Measurement was conducted continuously for two 36 h periods, each consisting of 12 h equilibrium and 24 h of data collection. The Data Grabber system, developed by Godby and Gregory (personal communication) was used to collect data pertaining to respiratory rates, oxygen consumption, and carbon dioxide and methane production. Animal heat production was calculated according to the following formula: heat production (kcal/d) = 4.89 x O_2 consumed (L/d) (McLean, 1972).

All samples for chemical analyses were dried at 60°C for 48 h and ground to pass through a 1-mm screen using a Wiley laboratory mill (model 4, Arthur H. Thomas Co.,

Philadelphia, PA), unless otherwise specified. Dry matter content of all samples was determined by drying ground samples at 105°C for 24 h. Nitrogen contents of feed and feces were determined with a nitrogen analyzer (LECO model FP-428, St. Joseph, MI). Approximately 100 mg of feed or feces were weighed in a tin foil cup which was folded, compressed into a tablet form and combusted completely. Nitrogen content was determined from the nitrogen concentration in the combustion gases. Crude protein contents of feed and feces were then calculated as N content x 6.25. Neutral detergent fibre (**NDF**), acid detergent fibre (**ADF**) and acid detergent lignin (**ADL**) were determined following the procedures described by Van Soest and Robertson (1980). Analysis of NDF was performed after preparing samples with a heat stable amylase (Sigma Chemical Co., St. Louis, MO) as suggested by Van Soest et al. (1991). Hemicellulose content was calculated as the difference between NDF and ADF and cellulose was the difference between ADF and ADL.

Variance analysis of data was performed with the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute Inc., 1990). Variation sources included in the initial model were Latin squares (n = 2), experimental periods (n = 4), ewes (n = 6), enzymes (n = 2), BES (n = 2) and their interactions. Because of missing data for one ewe in the fourth period of the experiment, least square means for main effects only were computed with the GLM procedure. The procedure of repeated measurements was employed to analyze the data that were sampled over time. Differences between main experimental treatments were determined using the method of the Student-Newman-Keuls when the variance component of the main treatment effect was significant ($P < 0.05$) with the F-test. Comparisons among individual effect of treatments were performed with the possible

difference procedure when interactions between treatments were significant ($P < 0.05$). Relationships between methane production, bacterial flow to the duodenum, passage rates, and ruminal digestion of fiber were predicted using the simple regression procedure. Coefficients of determination (R^2) and standard errors of estimates were calculated according to the method of Steel and Torrie (1980).

6.3. Results

6.3.1. α-Bromoethanesulfonic acid

Ruminal administration of BES did not change ($P > 0.05$) animal body weight during the experimental period (Table 6.1). Intakes of DM and CP were not influenced ($P > 0.05$) by ruminal administration of BES. Methane production was not depressed ($P > 0.05$) with BES treatment.

Ruminal administration of BES did not influence ($P > 0.05$) rumen pool sizes of either fluid or particulate matter (Table 6.1). Similarly, neither the rate of dilution of rumen fluid nor the fractional rate of passage of rumen particulate matter were altered by ruminal addition of BES (Table 6.1). Addition of BES to the rumen of sheep did not influence ($P > 0.05$) the outflows of DM, OM, NDF and ADF of grass hay to the duodenum. Similarly, the outflows of total N and DAPA to the duodenum were not changed ($P > 0.05$) with ruminal administration of BES (Table 6.1). Ruminal administration of BES did not influence ($P > 0.05$) the digestion of any nutrients of grass hay either in the rumen or post-ruminally (Table 6.2).

6.3.2. Exogenous fibrolytic enzymes

No difference in animal body weight was detected ($P > 0.05$) between the control and the enzyme treatment (Table 6.1). Pretreatment of grass hay with exogenous enzymes did

not affect ($P > 0.05$) intake of DM or CP. Methane production was not changed ($P > 0.05$) with enzyme treatment.

Pretreatment of grass hay with exogenous fibrolytic enzymes did not change ($P > 0.05$) pool sizes of rumen fluid or particulate DM (Table 6.1). Although enzyme treatment did not affect ($P > 0.05$) the rate of dilution of rumen fluid, it tended to increase ($P = 0.08$) fractional rate of passage of rumen particulate matter. There was no effect ($P > 0.05$) of exogenous fibrolytic enzymes on the flows of DM, OM, NDF, ADF and total N to the duodenum. Similarly, enzyme treatment did not increase ($P > 0.05$) flow of DAPA and hence rumen bacteria, to the duodenum. Pretreatment of grass hay with exogenous fibrolytic enzymes did not influence ($P > 0.05$) the digestion of DM, OM, NDF, ADF and N either in the rumen or post-ruminally (Table 6.2). Thus, the site and extent of digestion of grass hay was not changed ($P > 0.05$) by enzyme treatment.

6.3.3. Factors influencing methane production

Methane production per kg DMI decreased ($P = 0.04$, $R^2 = 0.25$) with the increases in DMI across individual sheep (Fig. 6.1). Total tract ($P = 0.09$, $R^2 = 0.18$) and ruminal ($P = 0.09$, $R^2 = 0.18$) apparent DM digestibilities both tended to increase with the increases in DMI (Fig. 6.2). Methane production per kg DMI decreased with the increases in total tract apparent DM digestibility ($P = 0.007$, $R^2 = 0.37$) and with the increases in ruminal apparent DM digestibility ($P = 0.007$, $R^2 = 0.38$) (Fig. 6.3 and 6.4). Similarly, methane production per kg DMI decreased with increases in total tract true DM digestibility ($P = 0.01$, $R^2 = 0.34$)

and with increases in ruminal true DM digestibility ($P = 0.006$, $R^2 = 0.38$) (Fig. 6.5 and 6.6). Both rumen fluid dilution rate ($P = 0.02$, $R^2 = 0.32$) and particulate matter passage rate ($P = 0.006$, $R^2 = 0.40$) increased with the increases in DMI (Fig. 6.7). Methane production per kg DMI tended to decrease ($P = 0.09$, $R^2 = 0.18$) with the increases in rumen fluid dilution rate, but methane production per kg RDOM was not significantly correlated ($P = 0.62$) with rumen fluid dilution rate (Fig. 6.8). Methane production expressed as either per unit of DMI or per unit of RDOM was not significantly correlated with rumen particulate matter passage rate (Fig. 6.9).

6.3.4. Relationship between methane production and bacterial yield

Methane production per kg OM apparently digested in the total tract decreased ($P = 0.0002$, $R^2 = 0.59$) with the increases in total tract apparent OM digestibility (Fig. 6.10). Similarly, methane production per kg OM apparently fermented in the rumen decreased ($P = 0.0001$, $R^2 = 0.82$) with the increases in ruminal apparent OM digestibility (Fig. 6.11). Diaminopimelic acid outflow per kg OM apparently digested in the total tract decreased ($P = 0.0001$, $R^2 = 0.76$) with the increases in total tract apparent OM digestibility (Fig. 6.12). Similarly, DAPA outflow per kg OM apparently fermented in the rumen decreased ($P = 0.0001$, $R^2 = 0.88$) with the increases in ruminal apparent OM digestibility (Fig. 6.13). Diaminopimelic acid outflow per kg OM truly fermented in the rumen also decreased ($P = 0.0001$, $R^2 = 0.71$) with the increases in ruminal true OM digestibility (Fig. 6.14). Diaminopimelic acid outflow per kg OM apparently fermented increased ($P = 0.0001$, $R^2 =$

0.74) with the increases in methane production per kg OM apparently fermented in the rumen (Fig. 6.15). There were no correlations between ruminal concentration of total VFA or molar percentage of VFAs and ruminal apparent OM digestibility (Fig. 6.16).

6.4. Discussion

6.4.1. α-Bromoethanesulfonic acid

Ruminal addition of BES did not depress ($P > 0.05$) methane production. This observation disagrees with the earlier in vitro studies (Chapter 4) and those of Martin and Macy (1985). The possible reasons that could cause the failure of BES to depress methane production in the present study have been discussed in detail in Chapter 5.

Ruminal administration of BES did not influence ($P > 0.05$) the rumen pool size of either fluid or particulate matter (Table 6.1). Similarly, neither the rate of dilution of rumen fluid nor the rate of passage of rumen particulate matter was altered by ruminal addition of BES (Table 6.1). Consistent with these findings, no changes were observed in outflows of any nutrients of grass hay and rumen bacteria to the duodenum with administration of BES (Table 6.1). To my knowledge, there has been no previous report concerning the effect of ruminal administration of BES on rumen pool sizes, passage rates and flows of nutrients and rumen bacteria to the duodenum.

Ruminal addition of BES did not affect either ruminal or post-ruminal digestion of any nutrients of grass hay. This is consistent with the findings in the total digestive tract (Chapter 5) and the in vitro study (Chapter 4).

6.4.2. Exogenous fibrolytic enzymes

Ruminal digestion of fibrous components and other nutrients of grass hay were not affected by enzyme treatment in the present study. This observation is consistent with the results obtained with cattle in our laboratory, in which supplementations of exogenous fibrolytic enzymes did not affect either in situ DM disappearance or total tract digestibilities of DM or NDF (Hristov et al., 1998b, in press). However, this observation is not consistent with previously reported positive responses in cattle (Feng et al., 1996; Lewis et al., 1996) and in vitro (Chapter 4). As reported in the previous chapter, although application of exogenous fibrolytic enzymes to grass hay increased activities of fibrolytic enzymes in the rumen fluid, fibrolytic enzyme activity associated with rumen particulate matter was not changed with enzyme treatment. Additionally, Hristov et al. (1998a, in press) found that the exogenous fibrolytic enzymes, which were of the same origin as used in the present study, were fairly resistant to rumen microbial degradation in vitro. Therefore, the observation that there was no response in ruminal digestion to the increased fibrolytic enzyme activities in rumen fluid was likely attributable to the weak binding of the enzymes with feed particles. Binding of enzymes to their appropriate substrates is an absolute prerequisite for the digestion of plant cell walls (McAllister et al., 1994).

In the present study, post-ruminal digestions of fibrous components were not increased with enzyme treatment. This observation is consistent with the results of Hristov et al. (1998b, in press), who found that pretreatment of a mixed diet containing rolled barley grain, corn silage and soybean meal with exogenous fibrolytic enzymes did not influence the

post-ruminal digestion of DM, NDF and N in cattle. They found that carboxymethylcellulase and β-glucanase activities of exogenous fibrolytic enzymes were not stable with respect to pepsin proteolysis and low pH in vitro. Therefore, the lack of improvement in the post-ruminal digestion of forage fiber with exogenous fibrolytic enzymes was attributed to the low resistance of exogenous fibrolytic enzymes to ruminal proteolysis and the low pH of the abomasum.

Pretreatment of grass hay with exogenous fibrolytic enzymes tended to increase the rate of passage of rumen particulate matter out of the reticulorumen, although it did not change the rate of dilution of rumen fluid (Table 6.1). The tendency to increase the rate of passage of rumen particulate matter with fibrolytic enzymes agrees with the observation of Feng et al. (1996), who found that pretreatment of dry grass hay with fibrolytic enzymes increased the rumen particulate passage rate in cattle. Fractional passage of particulate matter out of the rumen is determined in a larger part by the size of digesta particles in the rumen, i.e. smaller particles passing faster than larger ones (Ulyatt et al., 1986). Chewing during eating and rumination appear to be the principal means by which particle size of feed or digesta is reduced and microbial fermentation in the rumen per se has little effect on particle size reduction (Ulyatt et al., 1986). Its contribution to particle size reduction is likely to be in weakening cell wall structure by means of enzymatic digestion, thereby facilitating breakdown of particle size by chewing during rumination (Evans et al., 1974; Chai et al., 1984) and/or by detrition during digesta movement (Murphy and Nicoletti, 1984; Pond et al., 1987). A similar mechanism(s) would explain the tendency of increased particulate passage rate with exogenous fibrolytic enzymes.

Data from this study did not show any increase in outflows of any nutritive constituents or rumen bacteria from rumen to the duodenum with enzyme treatment (Table 6.1). These observations were not consistent with the tendency of fibrolytic enzyme to increase fractional particulate matter passage rate, which should have resulted in higher outflows of nutrients to the duodenum and lower ruminal fiber digestion and methane production. In addition, feed intake, which is often associated with increases in particulate matter passage rates, was not altered by enzyme treatment (Chapter 5). The tendency to which fractional passage rates of ruminal particulate matter were increased with exogenous fibrolytic enzymes was not determined in this study.

6.4.3. Nitrogen outflow

Total N flowing to the duodenum (27 g/d) was approximately 100% higher than total N intake (14 g/d) of sheep fed the fescue hay (1.4% N, 71% NDF, and 52% OM digestibility). Values for other nutritive constituent outflows and their partial digestion in the rumen or post-ruminally were within normal ranges. The extra gain of duodenal N observed in the present study is similar to the result reported by Weston and Hogan (1968), who found that total N arriving at the duodenum was 106% more than total N intake of sheep fed ryegrass with 0.96% N and 58% OM digestibility. Consistent with these observations, other researchers (Harris and Phillipson, 1962; Clarke et al., 1966; Hogan and Weston, 1967; Egan, 1974; MacRae et al., 1979) have also reported substantially more N (15-66%) arriving the duodenum of sheep than in the diet when sheep were fed other low-quality roughages.

The apparent gain in duodenal N would have been partially attributable to protein synthesis of rumen bacteria utilizing endogenous urea which enters the rumen via saliva (McDonald, 1948; Somers, 1961) and/or by diffusion from blood across the rumen wall (Houpt, 1959; Somers, 1961; Ash and Dobson, 1963). MacRae et al. (1979) measured transfer of endogenous urea N to the rumen to be 0.9-1.1 g N/d, which was equivalent to 14-22% of total N intake, in sheep given 460 g OM/d of low quality roughages. In contrast, Kennedy and Milligan (1978) estimated transfer of endogenous urea N at 7.3 g N/d in sheep given bromegrass hay. A similar value was reported by Varady et al. (1979), who found that 12.7 g urea N/d entered the rumen of sheep fed hay diet with supplements of wheat bran and barley. Kennedy and Milligan (1980) in a review pointed out that in sheep transfer of endogenous urea N to the rumen represented the equivalent of 7.5-26% of the dietary N intake. Applying these percentage estimates to the present study, availability of endogenous urea N to ruminal microbial protein synthesis would be less than 3.5 g N/d even when the highest value (26% of dietary N intake) was used in calculation. Therefore, contribution of endogenous urea N through bacterial protein synthesis to the duodenal N gain would be relatively small. These observations suggest that the extra duodenal N gain must have resulted from nitrogen sources other than endogenous urea N, which could have been either used for bacterial protein synthesis or passed directly into the duodenum. Possible sources of preintestinal endogenous protein are digestive secretions in the abomasum (Phillipson, 1964) and salivary protein (Hogan, 1975). In addition to nitrogen sources in the forestomachs, endogenous proteins in the form of enzymes, bile, mucus, serum albumin, lymph, epithelial cells and other degradable products from the small intestine lining would

also have contributed to the apparent gain of duodenal N. However, these parameters were not measured in the present study.

6.4.4. Methane production

6.4.4.1. Methane production per unit dry matter intake

Methane production averaged 22 g per kg of DMI (Table 1). This value is consistent with an average value of 21 g per kg of DMI estimated with sheep and goats (Sawyer et al., 1974; Joyner et al., 1979; Kreuzer et al., 1986; Aguilera and Prieto, 1991; Arieli, 1994) and a value of 22 g per kg of DMI estimated with cattle (Kirchgeβner et al., 1991).

When the results from individual sheep were examined, methane produced per kg DMI decreased with increases in DMI (Fig. 6.1). The negative relationship between methane production (g/kg DMI) and feed intake observed in this study agrees with the previous observations of Blaxter and Wainman (1961) and others (Johnson and Johson, 1995; Mathison et al., 1998).

One possible explanation for the decline in methane production per unit of food consumed with increasing intake could be a decline in digestibility which might be expected with increases in feed intake (Reid et al., 1980; Warner, 1981; Van Soest, 1982; Kennedy et al., 1986). However, this could not have been responsible for the decline in methane production in the present experiment since the apparent digestibility of DM in the rumen as well as the total tract tended to increase with intake (Fig. 6.2). In contrast, methane

production per kg DMI substantially decreased by 25 and 28% when total tract apparent DM digestibility increased from 35 to 55% and ruminal apparent DM digestibility increased from 18 to 30%, respectively (Fig. 6.3 and 6.4). This negative relationship between methane production and apparent digestibility is not consistent with previous conclusions that methane production per unit feed intake is not related to digestibility of the diet (Johnson and Johnson, 1995; NRC, 1996; Mathison et al., 1998). Increases in methane production per unit feed consumed with increases in digestibility have, in fact, been noted when sheep were fed at the maintenance level (Blaxter and Clapperton, 1965).

The apparent contradictions concerning interrelationships between apparent digestibility and methane production with feed intake arise because the relationships between apparent digestibility and intake is confounded by metabolic material in the feces at the low level of intake exhibited by the ewes (0.6-1.5 kg DM/d). When intake increases at below maintenance feeding levels, apparent digestibility can increase because of a reduction in the proportion of metabolic fecal DM in the feces (Van Soest, 1982). It is therefore more informative to examine the relationship of methane production with the true digestibility of the diet. When apparent total tract DM digestibility was adjusted for metabolic fecal outflows, which were estimated using an equation of Y (metabolic fecal materials) = 17.4 - 1.8 DMI as suggested by Van Soest (1982), to obtain estimates of the true DM digestibility, there was a significantly negative correlationship between true DM digestibility and methane production (Fig. 6.5). Similarly, when apparent ruminal DM digestibility was adjusted for bacterial DM outflow (estimated using a value of 3 mg DAPA per g bacterial DM as suggested by Czerkawski, 1986) to obtain estimates of the amount of DM truly degraded in

the rumen, there was a significantly negative correlationship between true ruminal DM digestibility and methane production (Fig. 6.6). It can therefore be concluded that declined methane production per unit of feed intake associated with increases in feed intake did not result from a reduction in feed digestibility but rather than other factors such as passage rates.

Generally, high intake results in increased passage rates of digesta out the reticulorumen (Owens and Goetsch, 1986), and increased passage rates of digesta are accompanied by a reduction in methane production (Stanier and Davies, 1981; Kennedy and Milligan, 1978; Okine et al., 1989). In agreement with these researchers, we found that both fluid dilution rates and particulate matter fractional passage rates were increased with increases in DMI across different ewes (Fig. 6.7) and that methane production per unit of feed intake tended to be reduced ($P = 0.09$) with increases in rumen fluid dilution rates (Fig. 6.8), although not significantly with particulate matter fractional passage rates (Fig. 6.9). The relatively weak response of methane production to passage rate of rumen particulate matter could have been associated with a low intakes of ewes (0.6 - 1.5 kg/d). It has been documented that increases in feed intakes have not consistently increased rumen particulate matter passage rates at low level of intake due to compensatory increases in ruminal volume (Galyean and Chabot, 1981; Colucci et al., 1982; Van Soest, 1982). Alternatively, methane productions may not have adequately reflected ruminal production since total digestive tract methane production rather than ruminal production measurements were made. Kennedy and Milligan (1978) reported that post-ruminal methane production accounted for 23% of the total methane produced in sheep therefore the rumen is a primary site of methane production in ruminants. Separate measurements of ruminal and post-ruminal methane production will,

however, be needed in future studies.

6.4.4.2 Methane production per unit of organic matter digested

The proportion of substrate ultimately reduced to methane actually decreased as digestibility increased. Thus methane production per kg of OM apparently digested in the total tract decreased by 56% when total tract apparent OM digestibility was increased from 40 to 60% (Fig. 6.10). Similarly, methane production per kg of OM apparently digested in the rumen decreased by 67% when ruminal apparent OM digestibility was increased from 16 to 39% (Fig. 6.11). We are unaware of any comparable in vivo experiments in which measurements of the amount of methane produced per unit of feed fermented in the rumen has been made, but Blaxter (1967) and Mathison (1990) both noted that methane production per unit of feed digested in the total tract declined with increases in total tract feed digestibility, although the declines were not as great as we noted in our experiment Thus, although our results were obtained with individual animals consuming the same feed, the results are consistent with differences obtained with differing feed qualities. Furthermore, it would appear that the changes in microbial populations and substrates being used by ruminal microorganisms, and which resulted in differences between digestibilities of individual animals, were more of a direct cause of variations in methane production per kg digestible OM than were changes in fractional passage or dilution rates since the latter were not related ($P > 0.05$) to the portion of digested organic matter which was fermented to methane (Fig. 6.8 and 6.9).

6.4.5 Bacterial growth

6.4.5.1 Diaminopilmelic acid production per unit organic matter digested

The amount of DAPA, hence bacterial protein, synthesized in the reticulorumen was not influenced by either fibrolytic enzyme or BES treatment (Table 6.1). However, DAPA outflow to the duodenum per kg of OM digested in the individual ewes, which is a measure of efficiency of bacterial growth, decreased with increased total tract organic matter digestibility (Fig. 6.12). In contrast, a summary of data presented in NRC (1996) suggests that in general no relationship is to be expected between efficiency of bacterial synthesis (g bacterial crude protein per kg dietary TDN) and total tract digestibility. It was acknowledged, however, that efficiency of microbial when expressed in this manner may be reduced when ruminants are fed diets based upon low quality forages, because the low fractional passage rates result in increased maintenance costs for the rumen microorganisms (Russell and Hespell, 1981) or when high concentrate diets are fed because of lower ruminal pH and slower microbial turnover.

The NRC (1996) suggests that ruminal digestible carbohydrate is the most accurate predictor of the efficiency of microbial synthesis. There was a marked negative relationship between the apparent digestibility of organic matter in the rumen and DAPA outflow per g of ruminal apparently digested OM (Fig 6.13). A similar relationship existed when it was expressed as OM truly fermented in the rumen (Fig. 6.14). There is evidence that forage-based diets, which have a relatively low digestibility, result in higher microbial yields (g/kg

OM fermented) than do concentrate-based diets which are more digestible (Mathers and Miller, 1981; Johnson and Bergen, 1982; Van Soest, 1982; Owens and Goestch, 1986). Moreover, the NRC (1996) suggests that maintenance requirements of non fibrolytic bacteria are higher than for those which degrade fiber. Although these observations apply mainly to differences between forage and grain based diets, they are consistent with our results that microbial efficiency (g/kg OM fermented) decreased with increasing feed quality.

6.4.5.2. Products of fermentation

It is of interest that both DAPA outflow from the rumen (g/kg RDOM), a measure of ruminal bacterial outflow, and methane production (g/kg RDOM) were negatively related to apparent ruminal OM digestibility (Fig. 6.11 and 6.13) and thus positively related with each other (Fig. 6.15). There were no changes in either molar ratio of propionate to acetate or concentrations of VFAs with ruminal OM digestibility in our study (Fig. 6.16). This would suggest that VFA production rates were also not related to ruminal OM digestibility since ruminal concentrations of VFAs are proportional to production rates (Owens and Goetsch, 1988). These observations are in disagreement with the general expectation that deceases in microbial synthesis efficiency (g cell DM/kg true digestible OM) are accompanied by increases in methane and/or VFA productions (Van Soest, 1982; Owens and Goetsch, 1988). Generally, lowered methane production per unit of feed digested with an increased proportion of concentrate in a diet is attributable to a rapid ruminal fermentation which favors propionate-producing microorganisms (Demeyer and Van Nevel, 1975). Rapid

ruminal fermentation is also often associated with a lower rumen pH, which may inhibit growth of methanogens and/or other hydrogen or formate producing microbes such as fibrolytic bacteria and protozoa (Demeyer and Henderickx, 1967; Eadie et al., 1970; Van Kessel and Russell, 1995). In the present study, rumen pH was not decreased ($P = 0.63$) with ruminal OM digestibility. I am therefore unable to explain the lack of a relationship between methane production and other rumen fermentation products in individual sheep in this study. This does raise questions concerning how the microbes operating in the rumen of sheep with a high ruminal OM digestibility dispose of electrons. One possible explanation could have been accumulation of ethanol, lactate or even hydrogen in the rumen. Unfortunately, these chemicals were not measured in this study.

The fact that there was a positive relationship between methane production and bacterial production per kg OM fermented appears to be in opposition to our hypothesis that methane production can be changed without influencing microbial efficiency. The results are, however, in agreement with the general concept that methanogenesis in the rumen benefits microbial synthesis (Wolin and Miller, 1988) by consuming hydrogen thereby facilitating regeneration of NAD^+ from NADH by fermentative bacteria (Hungate, 1975; Gottschalk and Andreesen, 1979). I am unaware of any research in vivo in which this concept has been examined previously. However, microbial growth is reduced when ionophores are included in ruminant diets concurrently with reductions in methane (Delfino et al., 1988; Johnson and Johnson, 1995; Mathison et al., 1998). The result is also consistent with studies with bacterial isolates (Chen and Wolin, 1979) and mixed ruminal bacterial populations (Eadie and Gill, 1971; Van Nevel and Demeyer, 1977; Hino, 1981; Henderson et al., 1981).

However the apparent relationship between methane production and microbial growth may not have universal application since in my earlier in vitro studies with the rumen simulation technique (Chapters 3 and 4) I demonstrated that inhibition of methanogen growth with canola oil, cod liver oil and BES, a specific methanogenic inhibitor, did not simultaneously inhibit the growth of ruminal cellulolytic or total fermentative bacteria. Unfortunately, BES failed to depress ruminal methanogensis in the present study so that we could not utilize this treatment as a means of evaluating the necessity of methane production for optimal microbial growth in the rumen as originally planned.

6.5. Conclusions and Implications

Pretreatment of grass hay with the fibrolytic enzymes had no effect on either ruminal or post-ruminal fiber digestion. Similarly, fibrolytic enzymes did not influence the rates at which rumen fluid or particulate matter passed through the reticulorumen or outflows of ruminal bacteria to the duodenum. Ruminal administration of BES did not influence the digestion process either in the rumen or post-rumen of sheep. It had no effect on the rates at which rumen fluid and particulate matter pass out of the reticulorumen or outflows of ruminal bacteria to the duodenum.

Regression analyses reveal that methane production per kg DMI decreased as DMI, passage rates and digestibility increased. These findings suggest that methane production per unit feed intake of ruminant animals can be reduced by the means of increasing feed intake and/or passage rates as well as digestibility. Diaminopimelic acid outflow per kg OM digested, hence rumen bacterial outflow to the duodenum, increased with increased methane production per kg OM digested, suggesting depression of ruminal methanogenesis could decrease availability of ruminal bacterial protein to ruminant animals. However, the question of whether depression of ruminal methanogenesis would have a serious detrimental effect on ruminal microbial synthesis was not answered in this study due to the failure of BES to depress methanogenesis.

Although it is important to reduce methane production in ruminant animals to improve efficiency of feed energy utilization and to minimize contribution to greenhouse gases, it is also important to consider any potential detrimental effect on ruminal microbial

protein synthesis if ruminal methanogenesis is depressed. A major benefit of ruminant production is that, the requirement for expensive protein supplements is reduced due to ruminal microbial protein synthesis in the rumen. Further in vivo research is require with a more potent anti-methanogenic agent(s) to examine the relationship between methane production and microbial growth in the rumen.

Table 6.1. Effects of exogenous fibrolytic enzymes and α-bromoethanesulfonic acid (BES) on ruminal digesta kinetics and nutrient flow to the duodenum of sheep fed grass hay

	Chemical[a]							
	None		BES			Probabilities[d]		
Item	CON[b]	ENZ[c]	CON	ENZ	SEM	BES	ENZ	BESxENZ
Body weight, kg	83.8	82.5	85.2	83.9	1.6	.40	.43	.99
Dry matter intake, kg/d	1.01	1.00	0.99	0.99	0.07	.87	.96	.96
Nitrogen intake, g/d	13.5	13.5	13.4	13.6	1.1	.99	.93	.91
Methane production, g/d	22.7	21.2	20.6	20.4	2.1	.44	.66	.76
Methane production, g/kg DMI	22.9	22.6	20.7	20.9	1.9	.35	.98	.91
Rumen pool size								
Fluid, L	5.3	5.6	6.0	5.7	0.5	.43	.94	.53
Particulate, kg DM	1.1	0.9	1.2	1.2	0.2	.31	.78	.56
Dilution and passage rates								
Fluid, %/h	8.2	8.7	8.2	8.5	0.5	.90	.39	.89
Particulate, %/h	3.9	4.6	3.7	4.1	0.3	.24	.08	.63
Nutrient flow to the duodenum								
DM, g/d	742	745	739	739	59	.93	.99	.98
OM, g/d	638	638	647	630	50	.99	.87	.87
NDF, g/d	409	419	399	395	36	.64	.94	.85
ADF, g/d	244	248	247	239	23	.90	.93	.80
Total N, g/d	26	27	27	28	5	.72	.71	.93
DAPA[e], mg/d	383	377	394	387	33	.74	.84	.99

[a]None: No α-bromoethanesulfonate treatment; BES: α-Bromoethanesulfonate treatment.
[b]CON: Untreated grass hay.
[c]ENZ: Grass hay treated with cellulase and xylanase.
[d]Probability that treatment has no effect; BES: main effect of α-bromoethanesulfonate treatment; ENZ: main effect of enzyme treatment; BESxENZ: interaction between α-bromoethanesulfonate and enzyme treatmeants.
[e]DAPA: Diaminopimelic acid.

Table 6.2. Effects of exogenous fibrolytic enzymes and α-bromoethanesulfonate (BES) on site of digestion in sheep fed grass hay

	Chemical[a]							
	None		BES			Probabilities[d]		
Item	CON[b]	ENZ[c]	CON	ENZ	SEM	BES	ENZ	BESxENZ
Apparent ruminal digestion, %								
DM	26.8	25.5	25.1	25.3	1.3	.47	.67	.58
OM	32.8	31.7	29.7	31.7	1.4	.24	.76	.30
NDF	42.2	40.4	41.3	42.4	1.6	.74	.83	.40
ADF	30.0	26.7	31.2	31.0	2.2	.22	.43	.52
N	-91.7	-101.3	-102.5	-106.1	6.3	.23	.30	.66
Apparent post-ruminal digestion, %[e]								
DM	25.0	24.2	24.4	23.7	1.7	.75	.65	.97
OM	30.9	30.2	30.5	29.8	1.9	.83	.70	.99
NDF	14.6	14.1	11.4	11.4	3.4	.39	.93	.94
ADF	15.9	14.6	16.2	14.9	3.7	.92	.72	.99
N	71.7	72.7	72.3	72.5	1.1	.85	.57	.76

[a]None: No α-bromoethanesulfonate treatment; BES: α-Bromoethanesulfonate treatment.
[b]CON: Untreated grass hay.
[c]ENZ: Grass hay treated with cellulase and xylanase.
[d]Probability that treatment has no effect; BES: main effect of α-bromoethanesulfonate treatment; ENZ: main effect of enzyme treatment; BESxENZ: interaction between α-bromoethanesulfonate and enzyme treatmeants.
[e]Percentage of nutrients flowing out of the rumen.

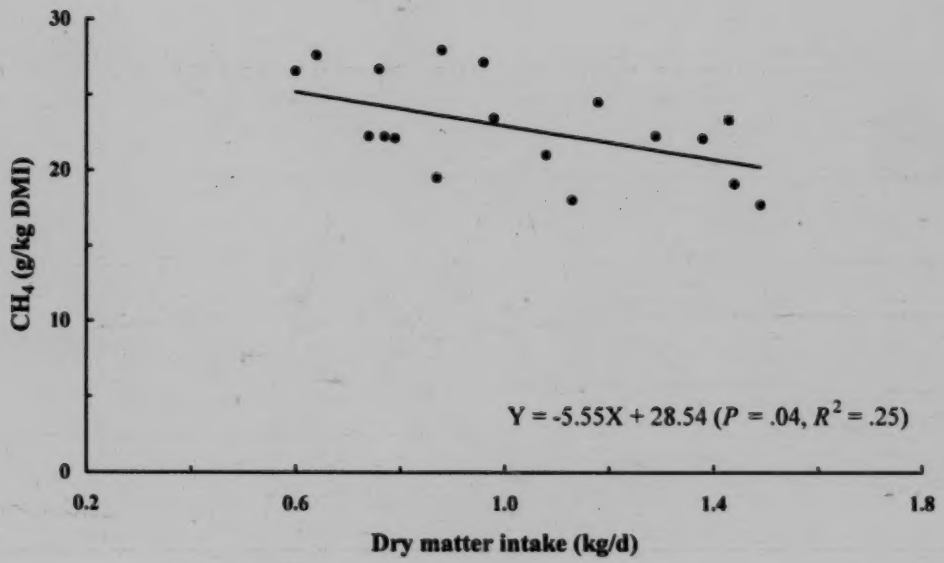

Fig. 6.1. The relationship between methane production and dry matter intake of sheep fed grass hay.

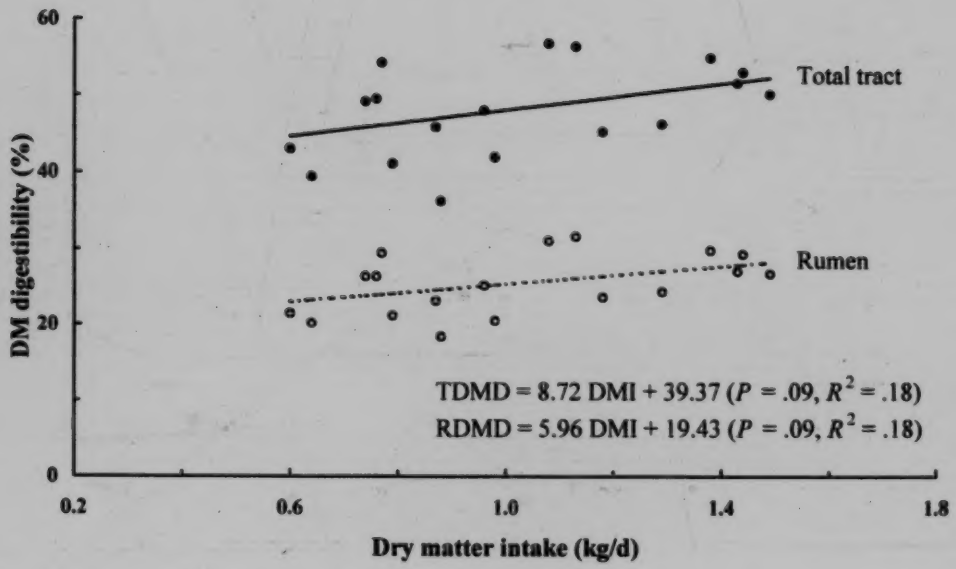

Fig. 6.2. The relationship between apparent DM digestibility and dry matter intake of sheep fed grass hay.

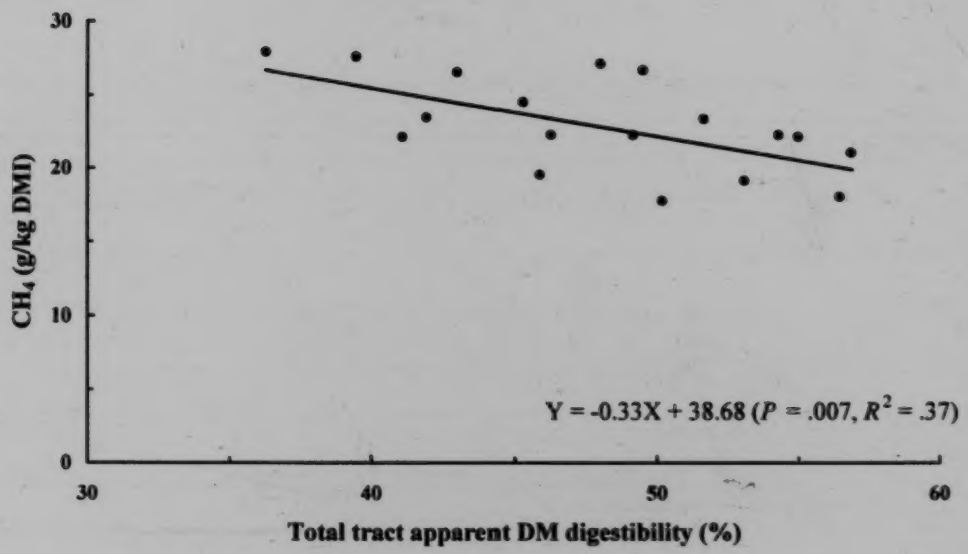

Fig. 6.3. The relationship between methane production and total tract apparent DM digestibility in sheep fed grass hay.

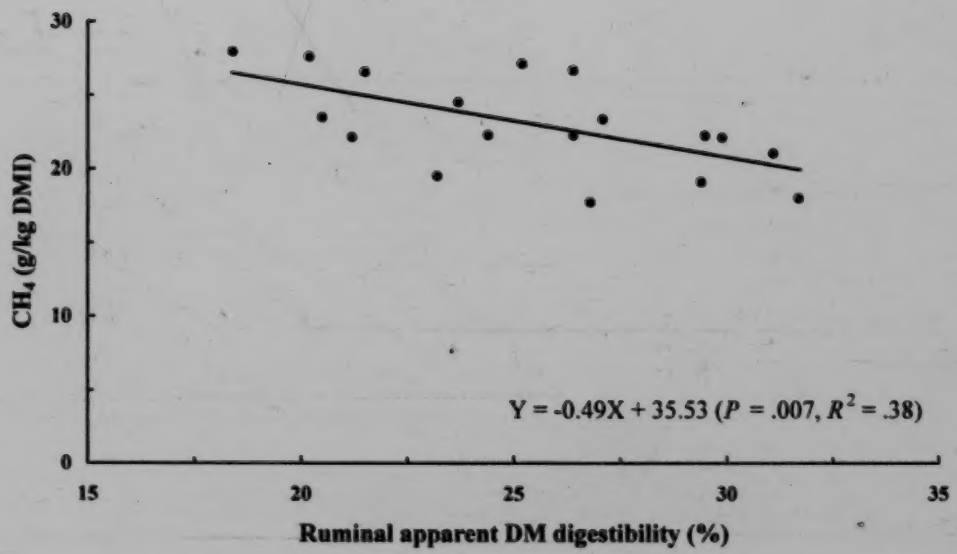

Fig. 6.4. The relationship between methane production and ruminal apparent DM digestibility in sheep fed grass hay.

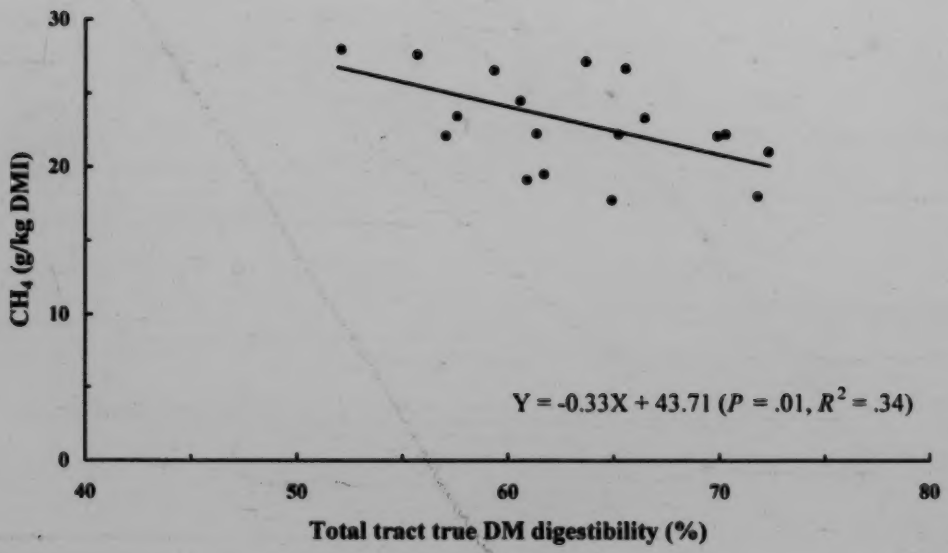

Fig. 6.5. The relationship between methane production and total tract true digestibility in sheep fed grass hay.

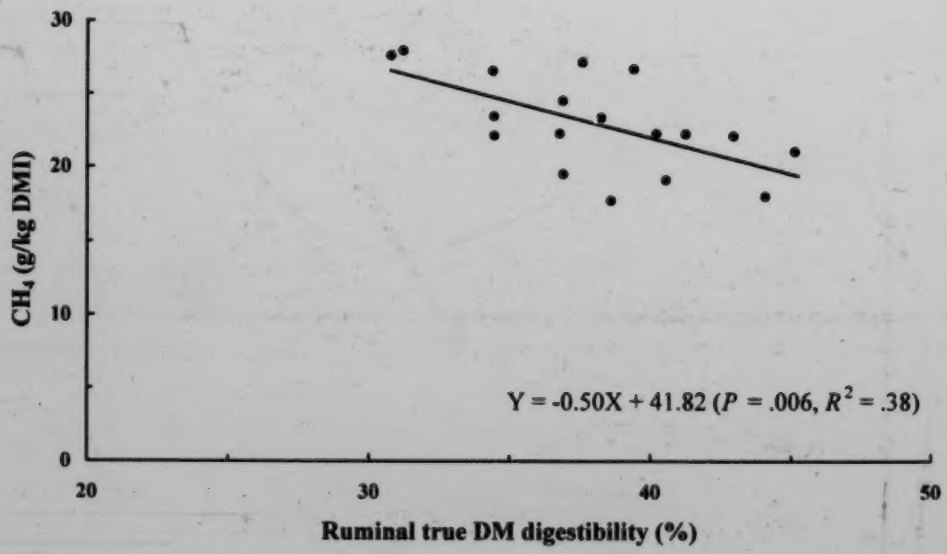

Fig. 6.6. The relationship between methane production and ruminal true digestibility in sheep fed grass hay.

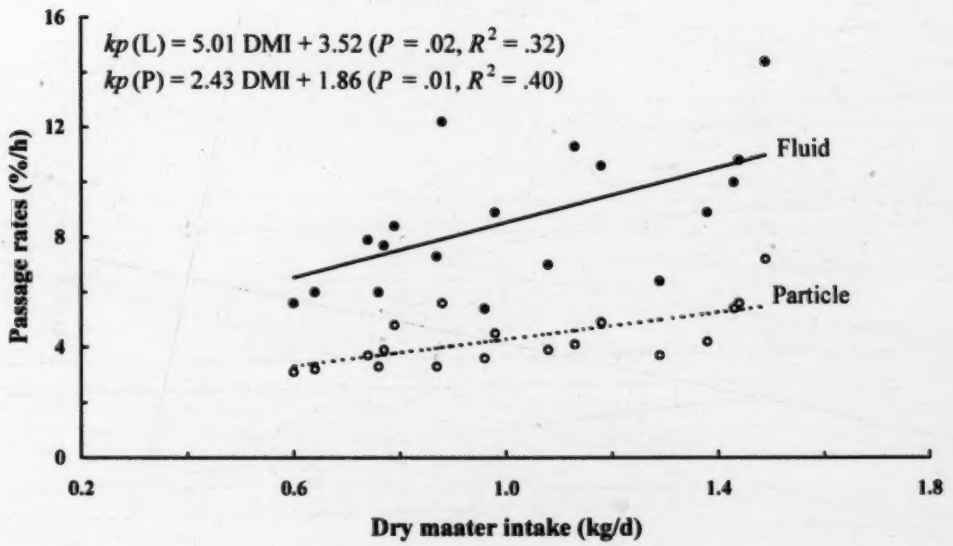

Fig. 6.7. The relationship between the rates at which fluid and particulate matter passed out of the rumen and dry matter intake of sheep fed grass hay.

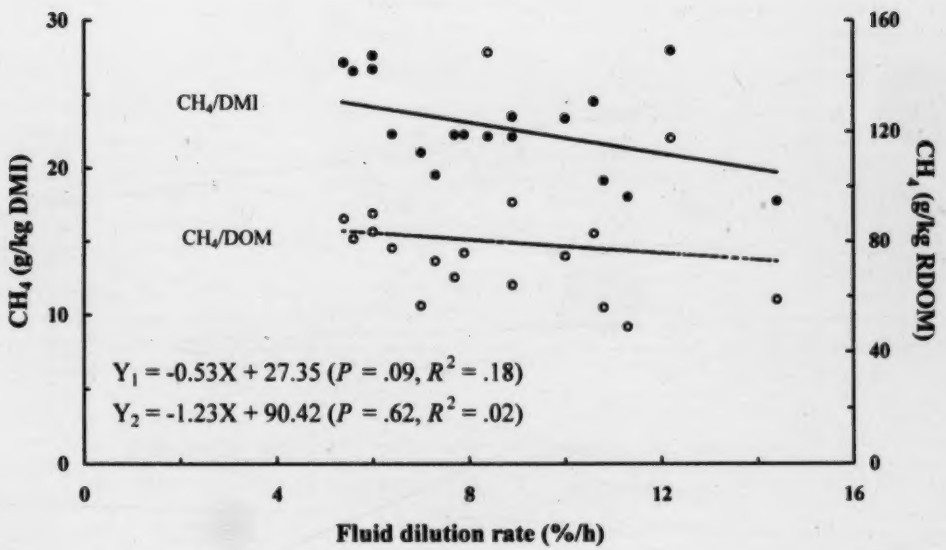

Fig. 6.8. The relationship between methane production and rumen fluid dilution rate in sheep fed grass hay.

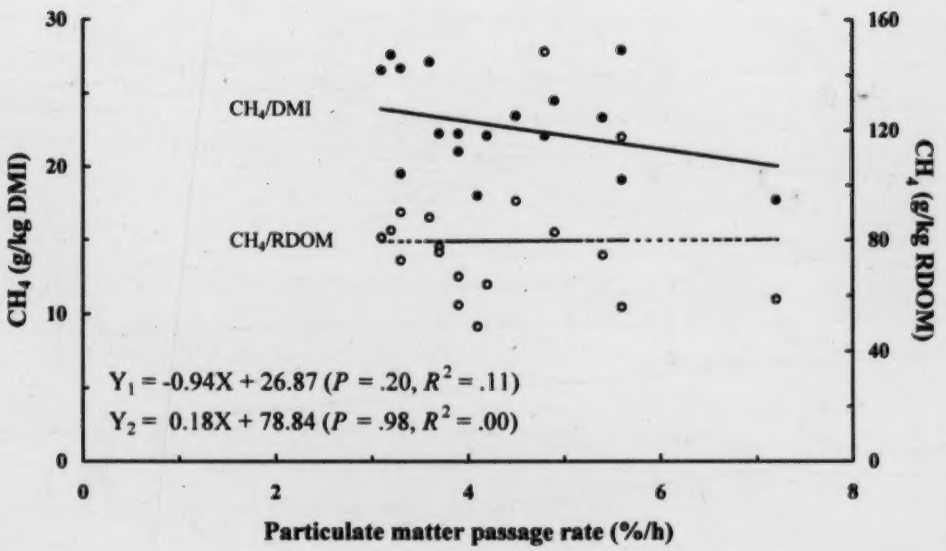

Fig. 6.9. The relationship between methane production and rumen particulate matter passage rate in sheep fed grass hay.

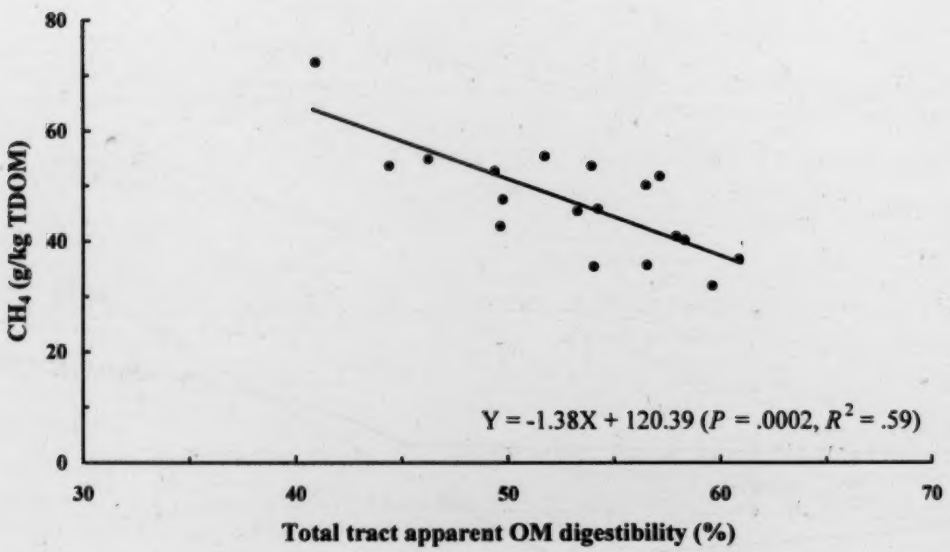

Fig. 6.10. The relationship between methane production and total tract apparent OM digestibility in sheep fed grass hay.

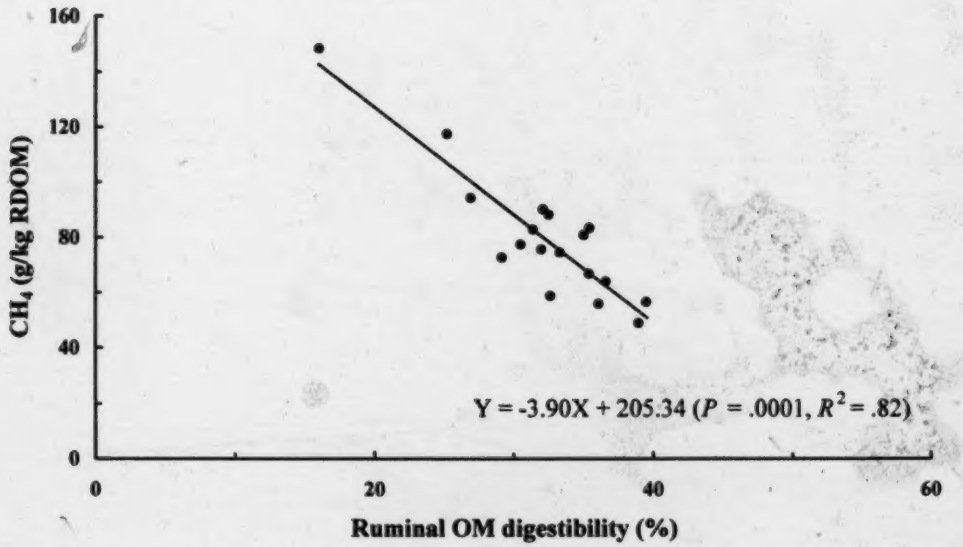

Fig. 6.11. The relationship between methane production and ruminal apparent OM digestibility in sheep fed grass hay.

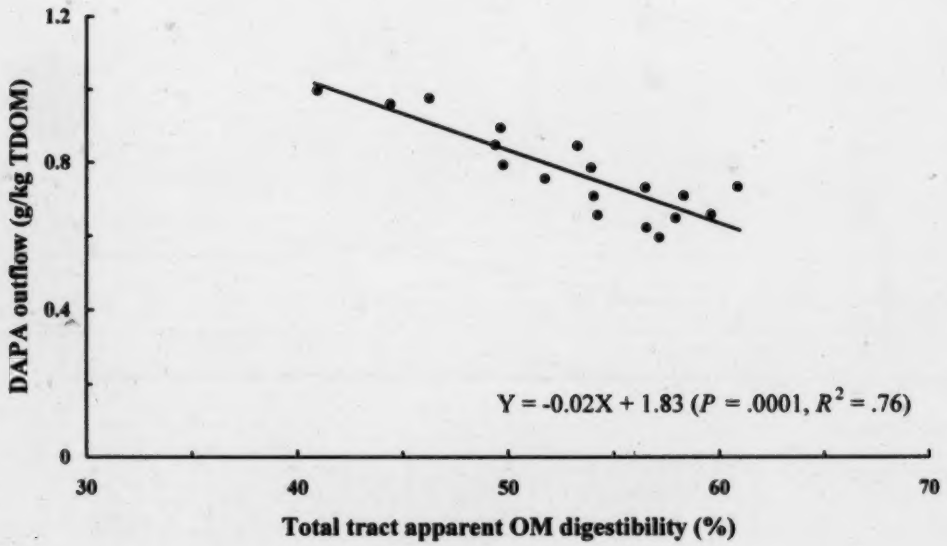

Fig. 6.12. The relationship between diaminopimelic acid outflow and total tract apparent OM digestibility in sheep fed grass hay.

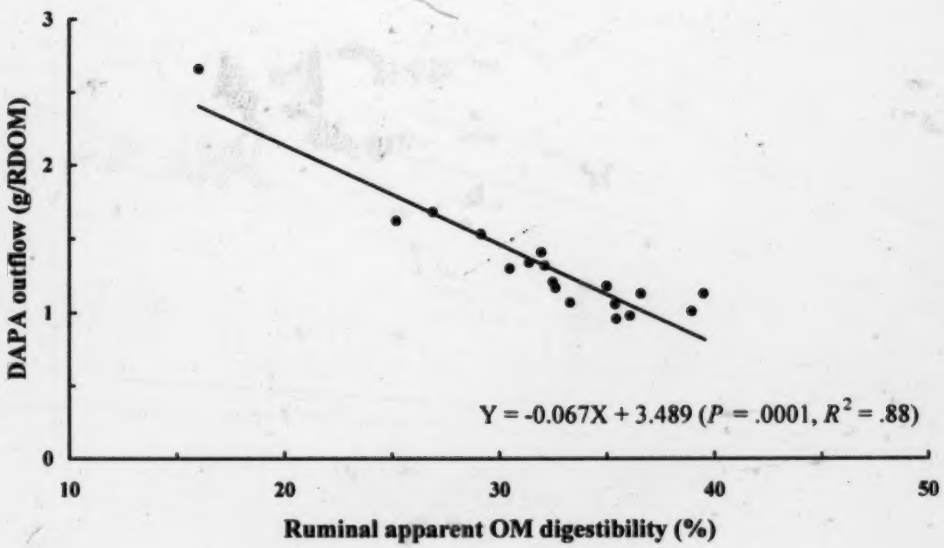

Fig. 6.13. The relationship between diaminopimelic acid outflow and ruminal apparent OM digestibility in sheep fed grass hay.

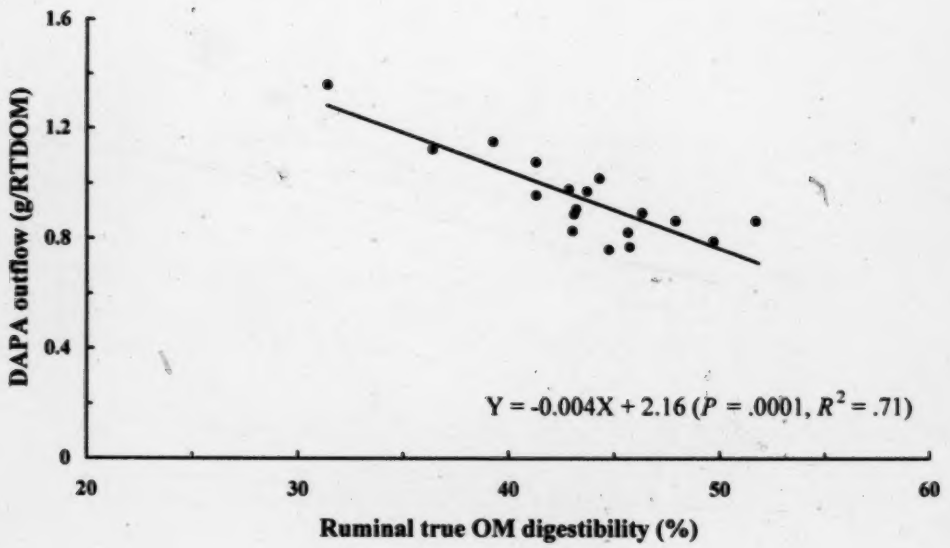

Fig. 6.14. The relationship between diaminopimelic acid outflow and total tract true digestibility in sheep fed grass hay.

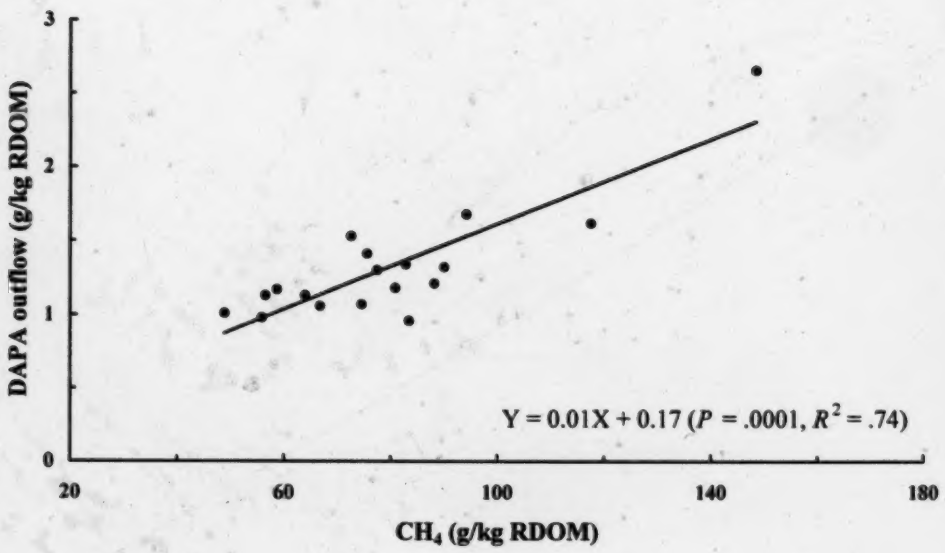

Fig. 6.15. The relationship between diaminopimelic acid outflow and methane production in sheep fed grass hay.

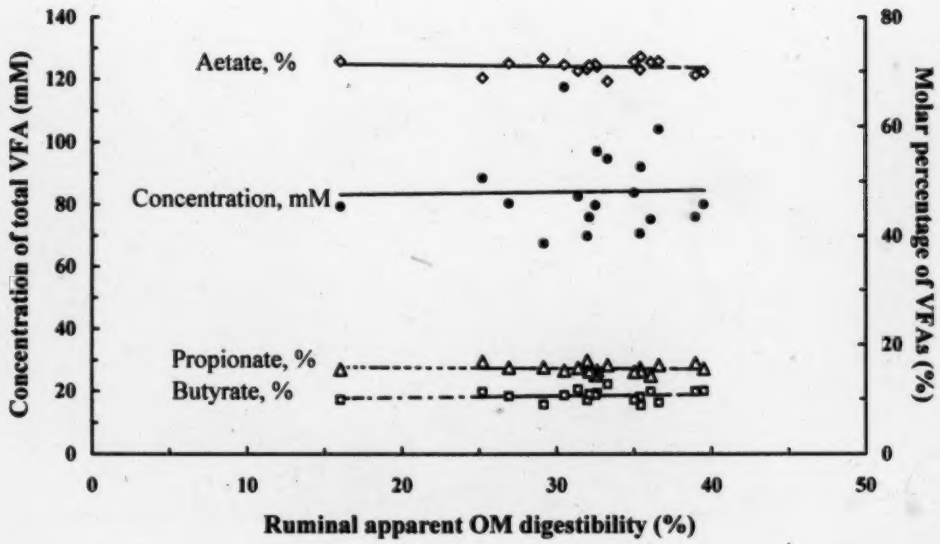

Fig. 6.16. The relationship between concentration of total volatile fatty acid, molar percentage of VFAs and ruminal apparent OM digestibility in sheep fed grass hay.

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7. GENERAL DISCUSSION AND CONCLUSIONS

7.1. Comparative Assessment of Experimental Systems

In the present thesis studies, the results of the animal study were in disagreement with the in vitro study regarding the effect of exogenous fibrolytic enzymes and α-bromoethanesulfonic acid on fibre digestion and methane production. The disagreement between two experimental systems could be attributable to the differences in distribution of microbial populations and dilution rates, although other factors such as methods used to prepare enzyme treatment cannot be ruled out.

Rumen protozoa were not successfully cultivated using the rumen simulation technique system (RUSITEC) in the present study. Similarly, Czerkawski and Breckenridge (1977) demonstrated that the protozoa, particularly the isotrichid holotrich species, declined rapidly in number and typically, were lost from the RUSITEC system after 7 d of normal operation. One factor which likely contributed to the loss of protozoa in the RUSITEC system is the daily opening of the vessels to change the feed bags. During feed change, the vessel contents are exposed to atmospheric oxygen. It has been observed that under normal operation, oxygen can dissolve in vessel contents and dissolved oxygen concentrations in vessel contents can reach levels of 5-10 μmol/l O_2 which are toxic to protozoa (Scott et al. 1983; Hillman et al. 1985). Another factor may have been lack of feed particles to which the slow-growing protozoa attach in order to avoid rapid wash out (Czerkawski and Breckenridge 1979). Hillman et al. (1991) demonstrated that addition of inert matrices into

the RUSITEC system prolonged the retention time of protozoa from 3 d to 7 d.

Loss of protozoa populations from the RUSITEC system could have been the cause of the disagreement concerning the effect of fibrolytic enzymes and BES on fibre digestion and methane production in comparison with the animal system. It has been well documented that lowermethanogensis is associated with the absence of protozoa (Itabashi et al. 1984; Whitelaw et al. 1984; Demeyer and Van Nevel 1979; 1986; Kreuzer et al. 1986). The absence of protozoa reduces total ruminal proteolytic activity (Stinchi et al. 1986; Ushida and Jouany 1985; Ushida et al. 1986). Lowered total proteolytic activity in RUSITEC system could have meant a reduction in degradation of exogenous fibrolytic enzymes, thereby increasing fibre digestion.

In addition to protozoa, methanogenic species resistant to BES may not have been present in the RUSITEC system. Absence of BES-resistant species would enhance the effectiveness of BES in depressing methanogenesis in the RUSITEC system.

Differences in dilution rate between two systems may also explain the disagreement between the in vitro and the in vivo systems. A fluid dilution rate of about 73% d^{-1} was observed in the RUSITEC system (Chapter 4), whereas the dilution rate of fluid in the rumen of sheep was about 200% d^{-1} (Chapter 6). The faster dilution rates in sheep could have resulted in washing BES and possibly enzymes out of the experimental system, reducing the effect of these agents on methane production. In addition to its effect on the effectiveness of anti-methanogenic agents, dilution rate per se functionally influences methane production through its effect on distribution of microbial populations and hence the extent of organic matter fermentation in the rumen. Stanier and Davies (1981) reported that a 50% decrease

in fluid dilution rates was associated with a 40% increase in methane production in an in vitro continuous culture. Kennedy and Milligan (1978) reported a 30% decline in methane production with 54 and 68% increases in ruminal passage rate constants of fluid and particulate matter, respectively, when sheep were exposed to cold without a change in feed intake. Similarly, Okine et al. (1989) observed that methane production was decreased by 29% when the mean retention time of particles in the rumen was decreased by 63% from 42 to 26 h when weights were placed in the rumen of cattle. My study with sheep also showed a tendency for methane production to be negatively correlated with fractional passage rates (Chapter 6).

In summary, although there are appreciable advantages in studying rumen functions with the RUSITEC system or other in vitro devices, e.g. lower cost and more rapid and reproducible in contrast with studies using animals, the RUSITEC system does have some aspects which deviate from the rumen. Undoubtedly, there will be future development and improvement not only in maintenance of methanogens and protozoa, but also in simulating the absorption of fermentation end-products, rumination and complex differential passage that occurs in the living animal.

7.2. Control of Methane Production in Ruminants

7.2.1. α-Bromoethanesulfonic acid

The data from the in vitro study of this thesis provides evidence that BES is a potent

specific inhibitor of methanogensis because a small amount (25 mM) depressed methanogenesis by 50% (Chapter 4). This result is consistent with the observations from an axenic culture with *Methanobacterium ruminantium* (Balch and Wolfe 1979) and a mixed culture of ruminal inoculum (Martin and Macy 1985). However, the potency of BES in depressing methanogensis was not expressed in the sheep study (Chapter 5). In addition, I could not detect any reduction in methane production during the first few days in unadapted sheep given BES (data not presented). This contrasts with results of Immig et al. (1996) who did observe BES inhibition of methanogensis in sheep for the first four days of treatment although the inhibitory effect of BES disappeared afterwards. Disagreement of in vivo studies with in vitro studies may be attributed to differences in the distributions of methanogenic bacterial populations sensitive to BES. Although information concerning this is limited, some studies have demonstrated adaptation of some methanogenic species to BES (Smith and Mah 1981). In addition, a strain of *Methanobacterium formicium* has been also discovered that is resistant to BES at a concentration of 0.2 mM without prior exposure to BES. Putting these findings together, the lack of response to BES in sheep may have been because BES resistant species and/or strains of methanogens were predominant in the rumen of sheep under conditions of the present study. These methanogens may not have been present in the RUSITEC system. Therefore, more research is needed to develop new techniques to discover new species and/or strains of methanogens which exist in the rumen and the hindgut of animals, and to study their physiology and ecology. Such studies may reveal more efficient means of controlling methane emissions without detrimental effects on feed digestion in ruminant animals.

7.2.2. Long chain unsaturated fatty acids

The data from this thesis study with the RUSITEC system (Chapter 3) showed that adding 10% lipids to diets reduced methane production from 26 to 85%, depending on the type of lipids and diets. This data provides further information to support the general conclusion that long-chain fatty acids can functionally depress methanogenesis (Czerkawski et al. 1966a, b, c; Czerkawski and Breckenridge 1969; Demeyer et al. 1969; Johnson and Johnson 1995; Mathison 1997). The huge variations, however, would suggest that different mechanisms exist for the inhibition of methanogenesis.

Johnson and Johnson (1995) in a review point out that decreased methane production is accompanied with decreased feed digestion when cattle or sheep are fed supplemental fat sources including tallow and soybean oil. They attribute this reduction in methane production with supplementation of animal fat or plant lipids to a reduced extent of feed fermentation rather than a direct effect on methanogenesis and concluded that dramatic decreases in methane production from supplemental lipids will only occur if digestion is inhibited. Data presented in this thesis disagree with conclusion of Johnson and Johnson (1995). Firstly, supplementation of coconut oil reduced methane production without any influence on digestion of the grain diet, although digestion of forage diet was reduced. Secondly, supplementation of canola oil or cod liver oil did not show any detrimental influence on digestion of either forage or high grain diet but did significantly reduce methane production. These observations were consistent with the distributions of microbial populations (Chapter 3). Czerkawski et al. (1966a, c) demonstrated that when stearic acid

was given to sheep fed dry grass hay, methane production fell by 29% with a 11% reduction in cellulose digestion. When oleic acid or linoleic acid was given to sheep, methane production fell by 39 and 43%, respectively, and cellulose digestion fell by only about 2%. Tamminga et al. (1983) found that lipid did not influence the digestion of starch or soluble carbohydrates. Putting the above observations together, it can be suggested that a reduction in methane production with supplemental lipids may not be necessarily accompanied with a decrease in dietary digestion. Whether or not lipids detrimentally influence ruminal fermentation of organic matter along with depression in methanogensis depend on the type of long-chain fatty acid in lipids, the type of diet and the distributions of microbial species in the rumen.

7.2.3. Exogenous fibrolytic enzymes

The data for the in vitro study of this thesis indicated that pretreatment of grass hay with exogenous fibrolytic enzymes enhanced methane production by 43% along with a 15% increase in cellulose and a 20% increase in hemicellulose digestion (Chapter 4). The observation of enhanced methane production disagrees with my original hypothesis that exogenous fibrolytic enzymes would reduce methane production by accelerating release of simple sugars (McHan 1986; Stokes 1992; Hristov et al. 1998a, in press), thus increasing the competitiveness of lactate- and/or propionate-producers with acetate-producers in the rumen.

Increased methane production with fibrolytic enzyme treatment in vitro was consistent with increases in the population of methanogens. It is interesting that the

increased population of methanogens associated with fibrolytic enzyme treatment was not accompanied by an increase in the populations of either cellulolytic or total fermentative bacteria. It is believed that no methanogens can utilize sugars, rather they utilize carbon dioxide, hydrogen, formate or other simple compounds which are generated as end-products by primary or secondary fermentative microbes (Zinder 1993). The reason for the increased population of methanogens which occurred with the exogenous fibrolytic enzyme treatment is not known. One possibility is that the enzymes contained some factors that were beneficial for the growth of methanogens. Further research is require to confirm these in vitro observations.

Unfortunately, results obtained with sheep did not confirm the in vitro results (Chapter 5). The possible reason for the failure of exogenous fibrolytic enzymes to improve fibre digestion and result in methane production in the rumen may have been due to the lack of binding of the enzymes with feed particles since binding of enzymes to their appropriate substrates is an absolute prerequisite for digestion of plant cell walls within the rumen (McAllister et al. 1994). The enzymes also failed to improve fibre digestion postruminally. This implies that free enzymes released from feed particles were degraded by proteolytic enzymes produced by rumen microbes (Vandevoorde and Verstraete 1987) and/or denatured by the low pH the abomasum (Hristov et al. 1998b, in press). Therefore, new techniques are needed, not only to screen potent enzymes, but also to reconstruct the molecular structure of the enzyme, which will improve its binding ability or enhance resistance to proteolysis and low pH.

7.3. The Role of Methanogenesis in Ruminal Degradation of Organic Matter

The data of this study in sheep demonstrated that DAPA outflow (g/d), a measure of ruminal bacterial outflow, increased with increases in methane production (g/d) (Chapter 6). Similarly, DAPA outflow per kg ruminal apparently digested OM (RDOM) increased with increased methane production per kg RDOM. These positive relationships between DAPA outflow and methane production are in agreement with the general concept that methanogenesis in the rumen or other anaerobic habitats is beneficial to fermentative microbial growth and hence fermentation of organic matter, because methanogens consume hydrogen thereby facilitating fermentative bacteria regenerating NAD^+ (Hungate 1975; Gottschalk and Andreesen 1979; Wolin and Miller 1988). A pure culture study has demonstrated that the cellulolytic microorganism *Ruminococcus albus* increases ATP generation from 3 to 4 moles per mole glucose fermented, with more acetate being produced and ethanol production being eliminated, when methanogens are present (Wolin and Miller 1988). In vitro studies with mixed rumen bacteria have shown that populations of ruminal fermentative bacteria and methanogensis change in the same direction when monensin is used as an anti-methanogenic agent (Van Nevel and Demeyer 1977; Chen and Wolin 1979; Henderson et al. 1981; Chapter 4). This relationship also holds true when coconut oil is used to reduce methane production in vitro (Chapter 3). Mathison (1997) demonstrated that in cattle methane production was reduced by 33% along with an 18% reduction in ADF digestion in cattle fed an 85% concentrate based diet. Similarly, Czerkawski et al. (1966c) reported that methane production fell by 29% with an 11% reduction in cellulose digestion

when stearic acid was given to sheep fed dry grass hay. Putting these observations together, it can be suggested that depression of ruminal methanogenesis would result in reductions in ruminal microbial protein synthesis and organic matter digestion in ruminants.

However, results from my in vitro studies with the rumen simulation technique, in contrast with the in vivo studies cited above, showed no inhibition of microbial growth accompanying inhibition of methanogenesis. For example, inhibition of methanogenic bacterial growth with canola oil, cod liver oil and BES, a specific methanogenic inhibitor, was not accompanied by inhibition of growth of ruminal cellulolytic or total fermentative bacteria (Chapter 3, 4). Reductions in methane production were not consistently correlated with changes in organic matter fermentation in vitro. Similarly, Czerkawski et al. (1966a) demonstrated that when methane production was depressed by 39 to 43%, there was only a 2% decrease in fibre digestion as long-chain unsaturated fatty acids were added to the rumen of sheep fed grass hay. These results would suggest that the ruminal fermentative microorganisms, including bacteria and fungi, were utilizing alternative reductive pathways for regeneration of NAD^+ from NADH rather than methanogenesis (Czerkawski, 1986). For examples, *Ruminococcus albus* can regenerate NAD^+ through a pathway of ethanol synthesis instead of H_2 formation (Miller and Wolin 1973). *Ruminococcus flavefaciens* can synthesize succinate using NADH as a reducing power (Latham and Wolin 1977). Similarly, some, if not all, ruminal fungi can also synthesize ethanol using NADH (Marvin-Sikkema et al. 1990). In addition to alternative utilization of NADH, some rumen bacteria can also use molecular H_2 or formate as energy sources. For instance, *Vibrio succinogenes* can reduce fumarate to succinate using formate or H_2 (Wolin et al. 1961). Also, *Desulfovibrio*

desulfuricans can oxidize H_2 and reduce sulfate to sulfide (Howard and Hungate 1976; Odom and Peck 1981). In addition, some rumen bacteria such as *Selenomonas ruminantium* and *Anaerovibrio lipolytica* can utilize H_2 and/or formate as electron donor and nitrate as electron acceptor (deVries et al. 1974).

Putting all these studies together, it can be suggested that whether or not the absence of methanogens influence ruminal fermentation will depend on the nature of the distribution of ruminal microbial species that rely on interspecies hydrogen transfer and the degree to which the microbial community and/or host animal adapt to utilize the end-products. For example, if the major substrate is cellulose and major microbial species is *Ruminococcus albus* and phycomycetes, then ethanol and lactate will accumulate in the absence of methanogens (Fig. 2.3 and Fig. 2.5). Similarly, if the major cellulolytic species is *Ruminococcus flavefaciens*, then a shift to succinate and thence propionate formation would be expected (Fig. 2.4). In this circumstance, hydrogen would continue to be generated through the pathway catalysed by pyruvate-ferredoxin oxidoreductase with NADH being used as reducing power for succinate formation.

Although the host animal helps to remove fermentative end-products, the interaction between microbes and host animal with the absence of methanogens has not been adequately studied. Since our attempt to ascertain the effect of depressing methanogenesis on microbial growth and hence organic matter digestion in the rumen was not successful due to the failure of BES to depress methanogensis in sheep, more in vivo research is required with a more potent anti-methanogenic agent(s) to examine the effect of the absence of methanogens on microbial growth and organic fermentation in the rumen of the intact animal.

7.4. Conclusions

Exogenous fibrolytic enzymes enhanced dietary digestibility and methane emission in vitro, but not with the intact sheep. Similarly, BES inhibited methanogenesis in vitro, but not in vivo. The reason for these discrepancies was not determined.

The in vitro study provides strong microbiological evidence that depression in methanogenesis with lipids is not necessarily accompanied by a reduction in dietary digestion. Whether or not lipids detrimentally influence ruminal fermentation depended upon the type of long-chain fatty acid in lipids, the type of diet, and the distribution of microbial species in the rumen.

These studies did not confirm or refute our major hypothesis that depression in methanogenesis would not have a detrimental on effect organic matter fermentation in the rumen of sheep, primarily due to the failure of BES to depress methanogenesis in the rumen. More in vivo research is required with a more potent anti-methanogenic agent(s) to examine the relationships between methanogenesis, microbial growth, and organic matter fermentation in the rumen of ruminant animals.

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